(1)

STUDIES CONCERNING THE PIERICIDINS

by

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PREFACE

The work described in this thesis was carried out in the School of Molecular Sciences, University of Warwick, Coventry, between November 1966 and October 1969. It is the original work of the author, except where specific acknowledgement is made, and has not been submitted for a degree at any other University.

The author wishes to make the following acknowledgements:

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CONTENTS

ABBREVIATIONS AND TERMINOLOGY

The following abbreviations are used in the text.

NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
ATP	adenosine-5^{1}-triphosphate
ADP	adenosine-5^{1}-diphosphate
P_i	inorganic phosphate
F_p	flavoprotein
Fe p	protein containing non-heme iron
Cyt.	cytochrome
EPR	electron paramagnetic resonance
pK_a	$-\log_{10}$ (dissociation constant)
ΔG^o	difference in Gibbs free energy
R	gas constant
T	temperature in degrees absolute
g., mg.	gram, milligram
l., ml., µl.	litre, millitre, microlitre (10^{-6} of a litre)
M, mM.	moles per litre, millimoles per litre
mm. Hg	millimetres of mercury (unit of pressure)
C	curie (unit of radioactivity = 3.7×10^{10} disintegrations per second)
mC, µC	millicurie, microcurie(10^{-6} of a curie)
c.p.m.	counts per minute
M.Pt.	melting point
B.Pt.	boiling point
^oC	degrees centigrade

T.L.C.	thin layer chromatography
R_f	$\frac{\text{rate of flow of sample}}{\text{rate of flow of solvent}}$
2,4-DNP	2,4-dinitrophenylhydrazone
DMSO	dimethylsulphoxide
HMPA	hexamethylphosphoramide
TAI	trichloroacecetylisocyanate
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
TCNE	tetracyanoethylene
lit.	literature value

The term "hydroxypyridine" is used in this dissertation to describe a compound in which the predominant tautomer is not specified. The tautomer of a hydroxypyridine bearing a proton on oxygen is referred to as a "pyridinol". The tautomer of a 2- or 4-hydroxypyridine bearing a proton on nitrogen is referred to as a "1-(H)-2-pyridone" or a "1-(H)-4-pyridone", and in general as a "1-(H)-pyridone". The tautomer of a 3-hydroxypyridine bearing a proton on nitrogen is referred to as the zwitterionic form of the 3-hydroxypyridine. The terms "pyridinol","1-(H)-2-pyridone" and "1-(H)-4-pyridone" are also used to describe hydroxypyridines existing predominantly in the tautomeric form specified.

ultra-violet spectra

UV	ultra violet
λ max	maximum of absorbance
nm	nanometre (10^{-9} of a metre)
ϵ max	molar extinction coefficient at

	maximum of absorbance
ϵ	molar extinction coefficient at given wavelength

infra-red spectra

IR	infra-red
ν (cm^{-1})	wavenumber of absorption (centimetre^{-1})
v.st., st., med., w	very strong, strong, medium, weak

nuclear magnetic resonance spectra

NMR	nuclear magnetic resonance
τ	tau value of peak in ^{1}H NMR spectrum
s., d., t., m	singlet, doublet, triplet, multiplet
J (H_z)	spin-spin coupling constant (Hertz)
MC/S	megacycles per second
ppm	parts per million
st., med., w	strong, medium, weak

mass spectra

m/e	atomic mass units per unit of positive charge
$(M)^+$	molecular ion
(M)	molecular peak (corresponding to molecular ion)
base peak	most intense peak of mass spectrum
I	intensity of peak in mass spectrum expressed as percentage of intensity of base peak

Peaks due to metastable transitions are quoted to two places of decimals. These values are calculated.

SUMMARY

The chemical similarities and contrasting biochemical properties of piericidin A and ubiquinone are presented and discussed. The former, a penta-substituted pyridine and a metabolite of Streptomyces mobaraensis, is an inhibitor of mitochondrial electron transport, whilst the latter is an electron carrier. A scheme to account for this difference is proposed involving an olefinic linkage of the long hydrocarbon side-chain. This was shown not to be responsible for the inhibitory action of piericidin A, since hydrogenation of the olefinic function did not noticeably reduce inhibitory potency.

Piericidin A was labelled with tritium in order to investigate its binding to mitochondria. It was found to be bound unspecifically in a linear manner up to 10 mμ moles/mgm. protein, but washing with bovine serum albumen removed most of this piericidin A. However a quantity corresponding to that required for the inhibition of NADH oxidation (0.02 mμ moles/mgm. protein) could not be removed in this way. It is concluded that this piericidin A was bound in a site specific manner. This specifically bound inhibitor was recovered unchanged on extraction of the mitochondrial lipids with acetone. Hence it is concluded that the inhibitor is not covalently bound to the mitochondria.

A number of analogues of piericidin A were synthesised. None of these compounds approached the potency of piericidin A as inhibitors, although those with lipophilic and phenolic properties were more effective in this respect than those without, suggesting the possible involvement of hydrophobic interactions and of a phenolic function during inhibition.

The possible tautomers of the proposed structure of piericidin A are considered in relation to its spectroscopic and chemical properties. Contrary to the behaviour expected from this structure, the molecule appears to exist as a pyridinol, rather than the 1-(H)-4-pyridone tautomer.

The evidence for the proposed structure of piericidin A is critically discussed. That for the arrangement of substituents on the pyridine nucleus depends partly on ultraviolet spectroscopic data, and more significantly, on two fragments arising from ozonolysis. The identity of one of these fragments (the 2,4-DNP of methyl pyruvate) is in doubt, since its properties differ from those of either of two fully characterised isomers of authentic material synthesised specially. It is concluded that piericidin A may be a β-hydroxypyridine, rather than the proposed ɣ-hydroxypyridine, since the remaining evidence is no longer conclusive. The spectroscopic properties of piericidin A mentioned above also favour this proposal.

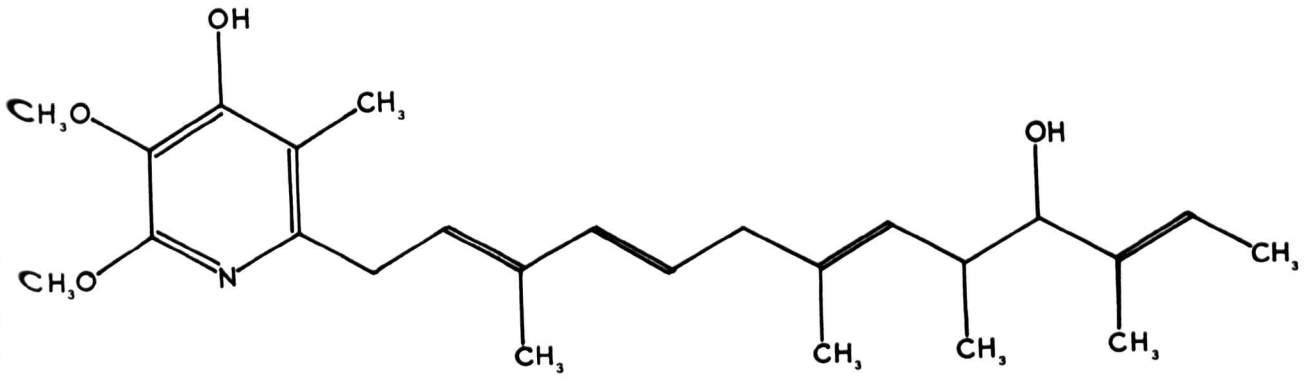
Two fully substituted β-hydroxypyridines, isomeric with the proposed pyridine nucleus of piericidin A, were synthesised. Neither had properties corresponding to piericidin A. With these and other hydroxypyridines, the comparative studies pertaining to piericidin A were extended, using the techniques of UV, IR, ^{1}HNMR and mass spectroscopy. A particularly simple diagnosis of hydroxypyridines by ^{1}H NMR spectroscopy was developed, involving the use of trichloroacetylisocyanate. In addition base catalysed deuterium exchange of protons in the methyl groups of substituted pyridines was studied.

As a result of these studies, distinctions can be made between β, and α or ɣ substituted methyl- and hydroxy-pyridines. Piericidin A appears to be a β-hydroxypyridine, its most likely structure being 2-alkyl-3,6-dimethoxy-5-hydroxy-4-methylpyridine.

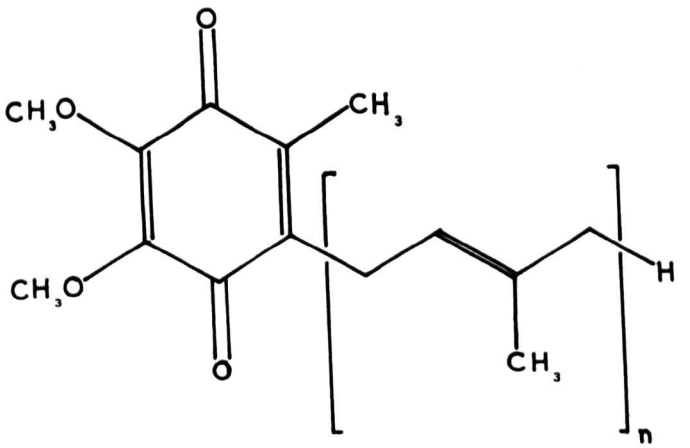
It is also concluded from the mass spectrum of piericidin A that there is an olefinic bond between carbon atoms 5 and 6 of the side-chain, rather than between carbon atoms 4 and 5 as published.

INTRODUCTION

FIGURE 1



PIERICIDIN A



UBIQUINONE $[n = 1-10]$

BIOCHEMICAL INTRODUCTION

GENERAL INTRODUCTION

Piericidin A is an insecticidal metabolite of Streptomyces mobaraensis, and was first isolated in 1963[1]. The chemical structure of piericidin A has been proposed as a penta-substituted pyridine ring[2,3], in which the substitution pattern closely resembles that of the ubiquinones (see figure 1). The possibility of tautomerism to a 1-(H)-4-pyridone enhances the similarity. The differences between the two structures can be considered as twofold.

(i) Piericidin A is a hydroxypyridine rather than a quinone.

(ii) In contrast to the ubiquinones, piericidin A has a non-isoprenoid side chain.

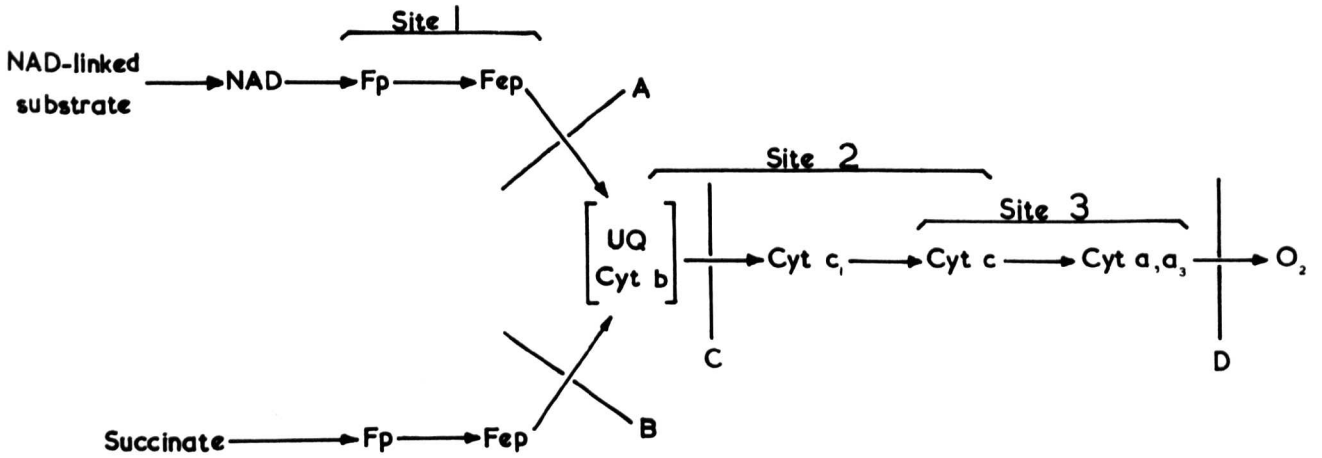
It was found that piericidin A inhibits mitochondrial reactions in the region where ubiquinone is involved. Inhibition of succinate dehydrogenase appeared to be competitive with ubiquinone, and potent inhibition of NADH dehydrogenase, which was not reversible by additions of ubiquinone, was also found[4]. These structural and biochemical contrasts initiated the present investigation.

The theories of electron transport and oxidative phosphorylation in mitochondria have been reviewed[5-7]. Of particular interest to this thesis are the possible involvements of ubiquinone[8-10], and of piericidin A and related inhibitors, in these processes.

The energy generated in respiring cells during the oxidation of substrates, principally of fatty acids, can be coupled to the synthesis of ATP from ADP and P_i. This conservational transduction of oxidative energy into the chemical energy of the triphosphoryl group of ATP is

FIGURE 2

THE MITOCHONDRIAL RESPIRATORY CHAIN



KEY

Fp	flavoprotein
Fep	protein containing non-heme iron
UQ	ubiquinone
Cyt	cytochrome
Sites 1,2 and 3	sites of oxidative phosphorylation
A,B,C and D	sites of action of particular inhibitors

SITE	INHIBITORS
A	piericidin A , rotenone , amytal
B	theonyltrifluoroacetone
C	antimycin A , 2-heptyl-4-hydroxyquinoline-1-oxide
D	cyanide ion , carbon monoxide

known as oxidative phosphorylation. In most types of cell, other than bacteria, the enzymes concerned with the generation of this oxidative energy, and the enzymes of oxidative phosphorylation, are a closely associated part of the inner membrane of highly organised cytoplasmic particles known as mitochondria. In bacteria, which have no mitochondria, they are bound to the cell membrane. The associated enzymes of the tricarboxylic acid cycle, and certain other oxidative functions, also appear to be localised in the mitochondria.

On the molecular level, most schemes for substrate oxidation in mitochondria postulate sequential electron flow through a series of carriers of increasing redox potential, terminating in molecular oxygen.

During the passage of a pair of electrons from substrate to oxygen, one molecule of ATP may be synthesised from ADP and P_i at a maximum of three coupling sites. A simple representation of the sequence of components of the mitochondrial respiratory chain appropriate to many systems examined, is made in figure 2. The approximate locations of coupling to oxidative phosphorylation, and of interruption of electron flow by inhibitors are also shown. The experimental evidence for all these assignments is reasonably consistent and will not be dealt with here. This discussion will concentrate on experiments dealing with the following topics:

(i) Oxidative phosphorylation

(ii) The function of ubiquinone

(iii) Inhibition by piericidin A

OXIDATIVE PHOSPHORYLATION

The problem of oxidative phosphorylation should first be considered from its standpoint as a biochemical phenomenon. The native mitochondrial process has not yet been observed in a soluble system, free from membrane structure. A reasonable conclusion from this is that a membrane structure is an inherent part of oxidative phosphorylation. The three coupling sites have not yet been assigned to specific redox reactions. This makes chemical formulation of the phenomenon difficult. There is good evidence that oxidative phosphorylation proceeds via a high energy intermediate.

(i) The existence of other energy dependent processes

Neither the respiration dependent accumulation of inorganic cations[11,12], nor the succinate linked reduction of acetylacetate[13], and α-ketoglutarate[14], are inhibited by oligomycin, an antibiotic which blocks ATP synthesis[15,16]. Furthermore certain submitochondrial particles, low in adenine nucleotide and P_i content, are able to utilise respiratory energy for other energy dependent reductions, although of course they are not able to synthesise ATP without added ADP and P_i[17,18]. It can also be concluded from this result that any primary coupling reaction does not involve P_i. The above results, together with the information that under certain conditions competition between ATP synthesis and other energy dependent reactions is observed[19-21] suggests the existence of a common high energy intermediate. Suppression of any of these processes in mitochondria causes an inhibition of substrate oxidation[22]. This so-called "respiratory control" is believed to be caused by a builk up of the high energy intermediate.

(ii) Loss of respiratory control

A diversity of chemicals, known as "uncouplers", cause inhibition of all energy dependent reactions, without stopping respiration[23-25]. The same effect may be brought about by structural damage to the mitochondrion. The cause of this "uncoupling" is consistent with the spontaneous decomposition of a high energy intermediate. Uncouplers are proposed to act on the respiratory chain side of the rate determining step of oxidative phosphorylation, following studies on the "ATP jump"[26]. This is observed, after the addition of ADP to respiring mitochondria, preincubated with P_i, as an initial fast synthesis of ATP superseded by a slower steady state rate. The initial "ATP jump" is not effected by uncouplers, whereas the steady state synthesis is abolished. Furthermore, competition between ATP synthesis and the uncoupling reaction has been shown, by measuring phosphorylation rates at different respiratory rates in the presence of fixed concentrations of uncoupler[27]. The competition is believed to be for a common high energy intermediate, and also explains the observation that phosphorylating efficiency increases with respiratory rate.

(iii) The chemistry of ATP synthesis and exchange

The following isotopic exchanges have been observed in intact mitochondria[28].

(a) The radioactive label of (^{32}P)-P_i is found in ATP after incubation with mitochondria in the presence of P_i, ADP and ATP.

(b) The radioactive label of (^{14}C)-ADP or (^{32}P)-ADP is found in ATP after incubation with mitochondria, without the

addition of P_i.

(c) The radioactive label of (^{18}O)-water is found in P_i and ATP after incubation with mitochondria. Similarly the radioactive label of (^{18}O)-P_i is found in water after incubation with mitochondria.

(d) The triphosphoryl oxygen bridge in ATP is supplied by ADP.

These observations are consistent with the following equilibria:

$$P_i + I^* \rightleftharpoons I \sim P + H_2O$$

$$ATP + I \rightleftharpoons I \sim P + ADP$$

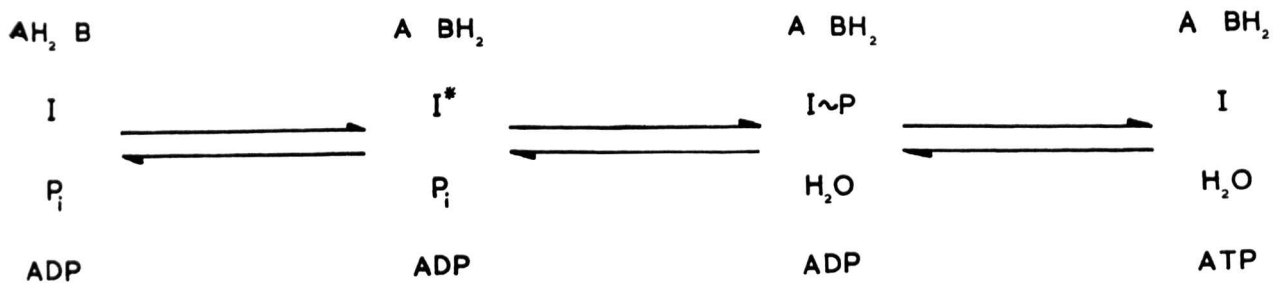
where I, I^* and $I \sim P$ are respectively non-energised, high energy and phosphorylated states of an undefined intermediate. Since the oxygen of P_i appears to originate from H_2O, and that of the triphosphoryl oxygen bridge of ATP from ADP, rather than from the intermediate in either case, no isotopic exchange into the intermediate would be detectable, except of course in its phosphorylated state.

(iv) Coupling factors[5]

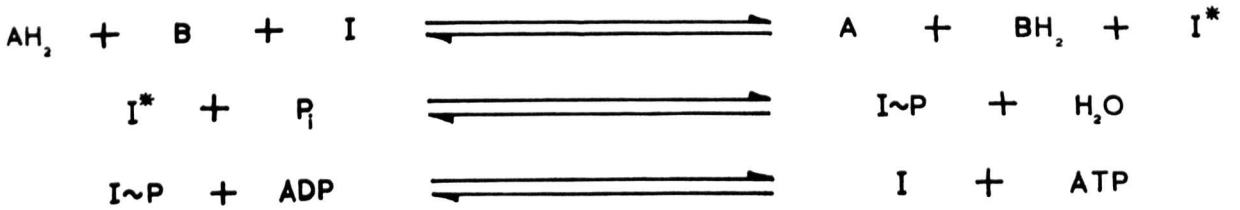
Certain isolable mitochondrial proteins, when added to specifically depleted mitochondrial or sub-mitochondrial preparations, increase their capacity to carry out oxidative phosphorylation. The identity of these "coupling factors" is diverse. The ability of certain of these preparations to catalyse the ATP exchange reaction in vitro can be chemically destroyed, without loss of the property of restoring mitochondrial oxidative phosphorylation[29]. They are thus believed to act indirectly via a structural modification of the mitochondrial membrane, rather than in a direct catalytic manner on a

FIGURE 3

A SCHEME OF OXIDATIVE PHOSPHORYLATION



or more conventionally



other proposals

i) the reaction $I^* \longrightarrow I$ is catalysed by uncouplers.

ii) I^* is the energy-source for other energy-linked reactions.

iii) Oligomycin inhibits further reaction of I~P.

KEY

A, AH_2, B and BH_2	unspecified respiratory carriers in oxidised and reduced forms
I, I^* and I~P	non-energised, energised and phosphorylated forms respectively of an unspecified intermediate

reaction of oxidative phosphorylation. The existence of these protein coupling factors emphasises the structural and steric sensitivity of the reactions and presumed intermediates of oxidative phosphorylation.

It is concluded that the isoenergetic sequence presented in figure 3 is a minimum working hypothesis for the observed reactions of oxidative phosphorylation. It should be emphasised that the involvement of a respiratory carrier in any of the proposed intermediates, and the possible existence of more than two such intermediates (the two being I^{*} and $I \sim P$) is neither precluded nor proven.

THEORIES OF OXIDATIVE PHOSPHORYLATION

Whilst the evidence for the existence of high energy intermediates is convincing, that concerning their molecular nature is divergent and elusive.

Briefly there are three hypothetical treatments:

(i) Involving conformational changes of the mitochondrial membrane.

(ii) Involving electrochemical potential difference across the mitochondrial membrane.

(iii) Involving discrete chemical intermediates.

(i) The conformational treatment

Of the three theories this is the least amenable to *in vitro* experimentation. The original postulate, by Boyer[30], proposes as intermediates a high energy conformation of a protein, with the further possibility of a sulphur-acyl bond. Three clearly distinguishable configurations of the mitochondrial inner membrane have been equated with energy states by Green[31], who also proposes that there are no other intermediates. Such a clear-cut relationship between the configuration of the membrane and biochemical state is not found by other workers[32]. It is difficult to test such a proposal further.

The involvement of the sulphydryl groups of proteins in the formation of a phosphorylated intermediate has been suggested, following mitochondrial studies involving sulphydryl reagents[33]. The phosphorylation of ADP has also been observed *in vitro* during the oxidation of several biochemically significant systems containing sulphur by bromine in pyridine[34].

Intermediates involving protein sulphydryl or sulphur-acyl functions should strictly be considered as chemical intermediates. Even the high energy conformation of a protein can be considered to be the macro-molecular effect of discrete molecular-interations, and hence to be strictly a chemical intermediate. However this point of view does not reveal any easier experimental approach.

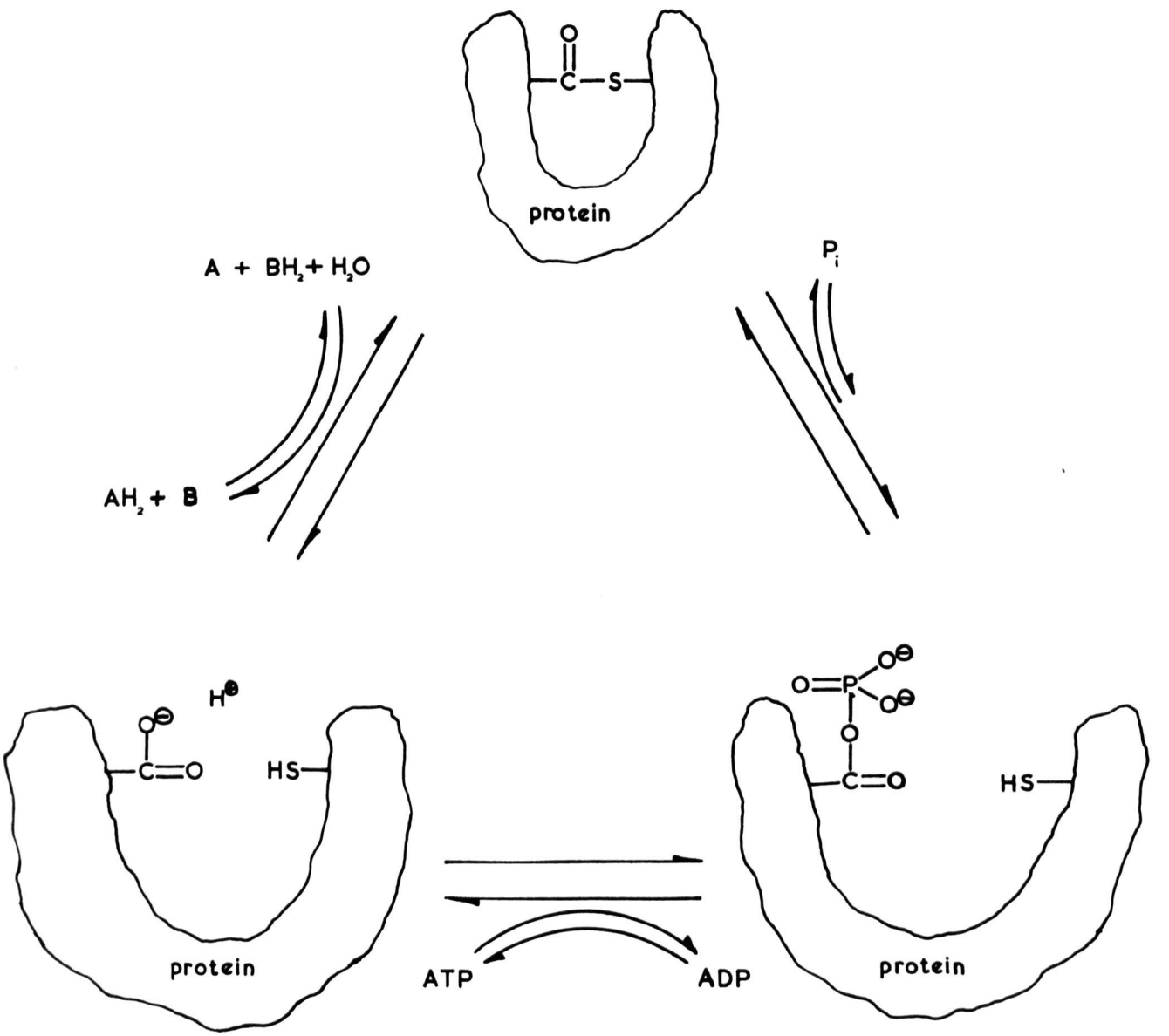
The effect of coupling factors, mentioned previously (page 11), lends support to the theory of different conformers as intermediates.

(ii) Membrane potential treatment

In the "chemiosmotic theory" of Mitchell, respiration causes proton transport across a membrane[35]. Evidence exists to this effect[35,36]. Thence a pH gradient and electrochemical potential difference is produced across the membrane. Reversal of this process is said to drive the formation of the anhydride between ADP and P_i, via a vectorial ATPase bound to the membrane. The mechanism of this process is an unsatisfactory part of the theory. Two strong points of the same are firstly the ready explanation of the necessity for a membrane, and secondly the explanation of the mechanism of action of uncouplers[37,38]. There is evidence that these chemicals increase the permeability of the membrane to protons, and hence collapse any membrane potential. The chemical diversity of uncouplers is not easily accounted for by other theories. Conversely the action of coupling factors may be explained by proposing that they decrease the permeability of the membrane to protons, stabilising any membrane potential.

FIGURE 4

A SCHEME INVOLVING PROTEINS IN OXIDATIVE PHOSPHORYLATION



[A, AH_2, B and BH_2 unspecified respiratory carriers in oxidised and reduced forms]

(iii) Chemical intermediate treatments

In chemical hypotheses the intermediate I^* (figure 3) is conceived as a relatively stable chemical substance having a high energy bond. The intermediate I~P is considered to have similar stability and energy, and to be capable of phosphorylating water or ADP.

Three different chemical species have been put forward as chemical intermediates at various times.

(a) Proteins[30]

(b) NAD[39]

(c) Quinones[40]

(a) Proteins

The idea of the involvement of the sulphydryl or sulphur-acyl functions of proteins as chemical intermediates has already been mentioned[30,33,34], (pages 13-14). One such scheme is presented in figure 4. The observed isotopic exchanges between ATP, ADP, P_i and water (pages 10-11) may all be explained on this scheme. Phosphorylation by the proposed acyl phosphate would result in the retention of the anhydride oxygen by the acyl function[41]. The other reactions, involving sulphur, are chemically demonstrable[34,42]. The difference between the generalised scheme in figure 3 and that in figure 4 lies in the sequence of the dehydration reaction.

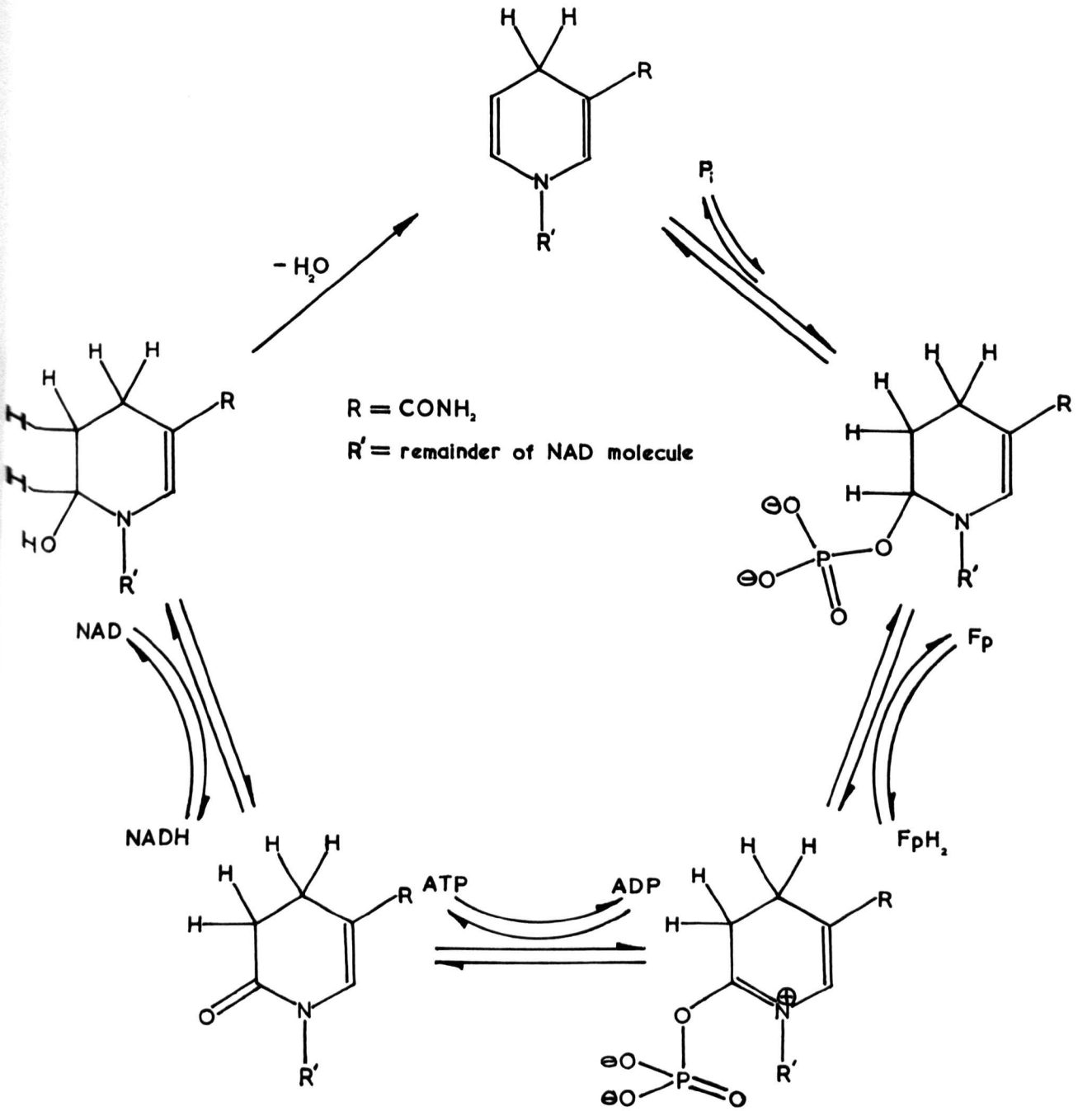
In figure 3: $I^* + P_i \rightleftharpoons I\sim P + H_2O$

In figure 4: $I + AH_2 + B \rightleftharpoons I^* + H_2O + A + BH_2$

Thus in the latter case the observed isotopic exchanges between

FIGURE 5

A SCHEME INVOLVING NAD IN OXIDATIVE PHOSPHORYLATION



water, and P_i and ATP are less readily explained although they are perfectly feasible. The implied exchange of oxygen into the carboxyl function of the protein would be difficult to detect isotopically, since exchange into such functions will be considerable, irrespective of any oxidative phosphorylation.

Some phosphorylated proteins have been isolated, possessing many of the expected properties of a phosphorylated energy-transport intermediate[43,44]. These include:

(i) the ability to phosphorylate ADP,

(ii) the ability to catalyse the ATP $\rightleftharpoons$ ADP exchange reaction, and

(iii) the ability to act as coupling factors.

One author has even tentatively suggested that the phosphate function is an acyl phosphate[44].

It thus appears that this is one approach where chemical and biochemical deductions merge.

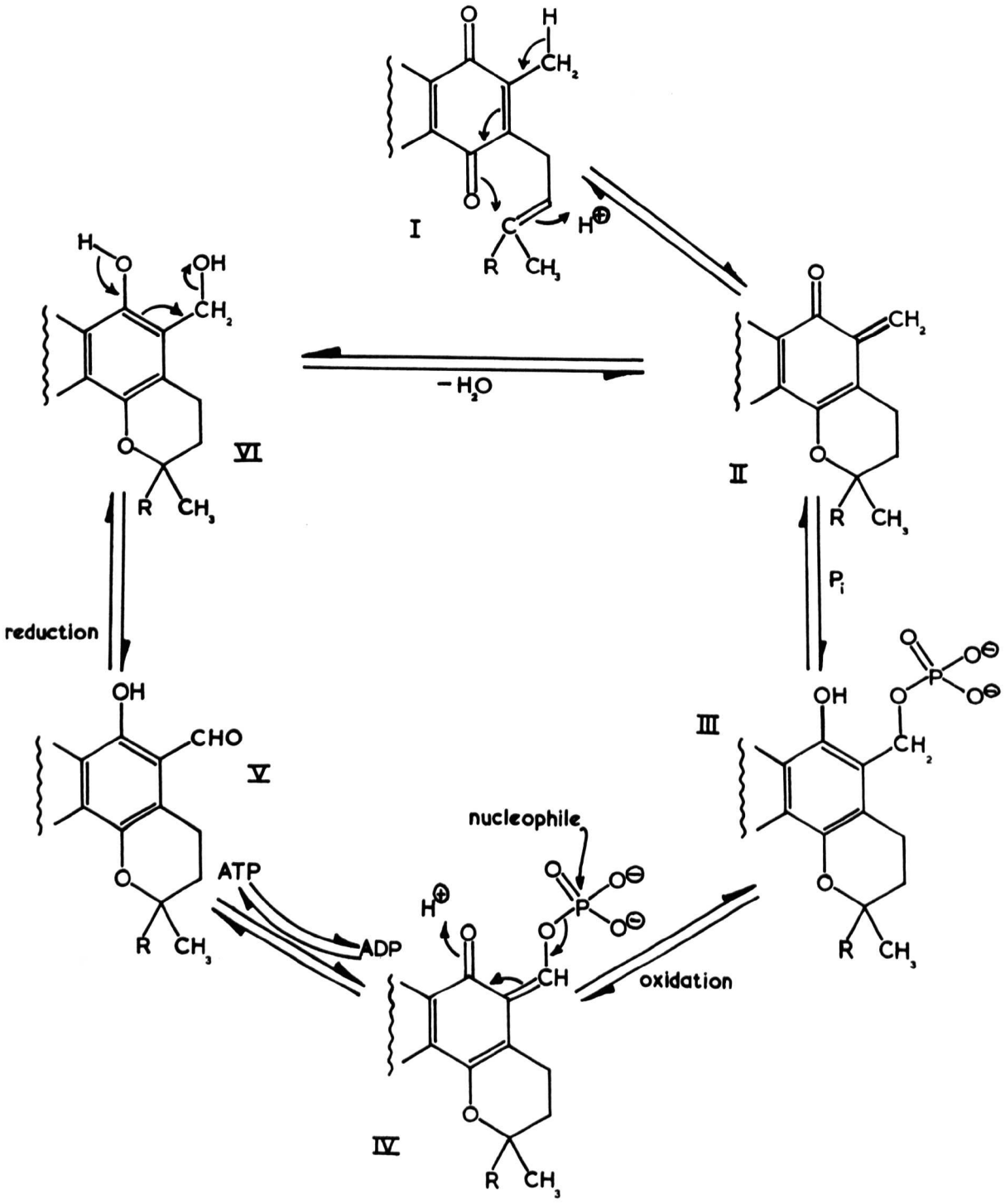
(b) NAD

The participation of NAD derivatives in oxidative phosphorylation has been suggested[39]. The scheme proposed is presented in figure 5.

Although NAD derivatives of unusual nature have been isolated[45,46], their chemical identity remains obscure. Furthermore, the existence of a high energy intermediate not involving adenine nucleotides or P_i has been inferred[17,18], (page 9). This is divergent with the proposed scheme.

FIGURE 6

A SCHEME INVOLVING QUINONES IN OXIDATIVE PHOSPHORYLATION



(c) Quinones

Following the observation that the oxidation of hydroquinone phosphates in vitro can lead to phosphorylation[47], schemes were suggested involving quinones in oxidative phosphorylation[40,48]. Of these the most chemically consistent is presented in figure 6[49]. An in vitro study of this scheme has been made[50], in the course of which evidence for the equilibrium I $\rightleftharpoons$ II has been found[51]. No evidence for the reactions II $\rightarrow$ III, III $\rightarrow$ IV and IV $\rightarrow$ V was found, and compound III proved to be very unstable. Considered from a biochemical angle, it should be pointed out that the primary high energy intermediate of this scheme, IV is phosphorylated. This is not very satisfactory, since it has already been deduced that it is unlikely[17,18](page 9). The direct involvement of ubiquinone in oxidative phosphorylation (as distinct from its involvement as a respiratory carrier) has never been demonstrated. Experiments in which the involvement of vitamin K in oxidative phosphorylation in Mycobacterium phlei has been demonstrated[52,53] are the only direct evidences for such an involvement for quinones in general. Indeed many experiments designed to detect the existence of quinone intermediates, using the technique of isotopic labelling, suggest their paucity[54,56]. The scheme presented in figure 6 is considered later in relation to a possible mechanism of action of piericidin A (page 26).

In concluding this consideration of chemical intermediates in oxidative phosphorylation, the following points should be considered.

(i) No chemical intermediate corresponding to I* (figure 3) has yet been identified.

(ii) Only in the case of proteins is there consistent evidence for the existence of an intermediate corresponding to I~P, and even in that case its chemical nature is obscure.

(iii) It might be expected, from the chemical standpoint, that the three "coupling sites" would possess intermediates of different chemical identity. No intrinsic differences in the three coupling sites have been established.

Thus the precise nature of the molecular processes of oxidative phosphorylation remains unsolved and is the focus of controversy.

THE FUNCTION OF UBIQUINONE

The function of ubiquinone as a mitochondrial electron carrier in the respiratory chain (figure 2) is established by the following observations.

(i) It is found in relatively high concentrations in all aerobic tissues tested[57-59].

(ii) Succinate dehydrogenase and NADH dehydrogenase activities lost following the extraction of ubiquinone from mitochondrial preparations, can be restored by the addition of ubiquinone[60-64].

(iii) Ubiquinone is reduced and oxidised during electron transport[59,65-68]. Some discrepancy has been observed between the rate of turnover of the quinone and the respiratory rate[68-70]. However other experiments show this rate to be consistent with the central position of ubiquinone in the respiratory chain[71].

(iv) Three of four isolated complexes of the mitochondrial respiratory chain react specifically with ubiquinone[72]. These four complexes can be reconstructed into a complete respiratory chain.

As mentioned previously (page 17), there is no direct evidence involving ubiquinone in mitochondrial phosphorylation reactions. Such an involvement may be argued by analogy with vitamin K in bacterial systems[52,53]. The necessity for an aromatic methyl group, and an adjacent $\beta\gamma$ unsaturated carbon side chain, has also been inferred, as a pre-requisite for quinones involved in oxidative phosphorylation, from such bacterial studies[52b]. However this may be disputed from the negative results of certain experiments involving isotopic exchange, designed to detect any quinone energy-transport intermediates[54-56].

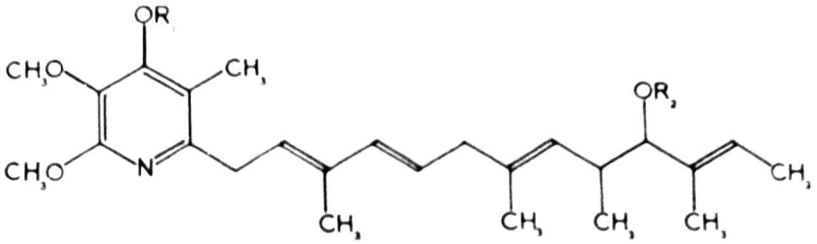
INHIBITION BY PIERICIDIN A

It was found that piericidin A inhibits the oxidation of NADH and of succinate in mitochondria, but that the NADH dehydrogenase system is several orders of magnitude more sensitive than the succinate dehydrogenase system[4, 73-75]. NADH dehydrogenase is inhibited by concentrations of piericidin A of the order of 3×10^{-5} μ moles/mg. of protein, and the inhibition is not reversed by the addition of ubiquinone. Succinate dehydrogenase is inhibited by concentrations of piericidin A of the order of 10^{-7} or 10^{-6}M, and the inhibition is reversed by the addition of ubiquinone, suggesting competition for a common reaction site. Evidence has also been produced to the effect that piericidin A can act as an uncoupler[74,75], and that at high concentrations it may inhibit cytochrome C oxidase[46,74,76].

Studies with radioactively labelled piericidin A[77-79], in agreeement with results presented in this thesis and elsewhere[80], have shown that an amount of the inhibitor, equivalent to the minimum required for complete inhibition of NADH dehydrogenase, is strongly bound to mitochondria. Concentrations above this may be easily removed by washing with bovine serum albumen. This removal is also consistent with the reversible inhibition of succinate dehydrogenase activity at higher concentrations. The binding of piericidin A to mitochondria bears an approximately linear relationship to the concentration of piericidin A added, over-all concentrations tested[78-80]. Between one and two molecules of piericidin A per unit of NADH dehydrogenase become strongly bound to mitochondrial preparations[78-81], more recent results favouring the higher figure[82].

FIGURE 7

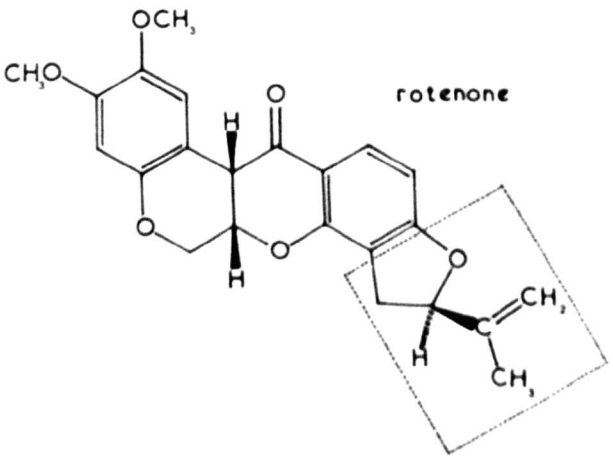
THE PIERICIDINS AND RELATED INHIBITORS



piericidin A $R_1 = R_2 = H$; diacetate $R_1 = R_2 = COCH_3$; monoacetate $R_2 = H$, $R = COCH_3$.

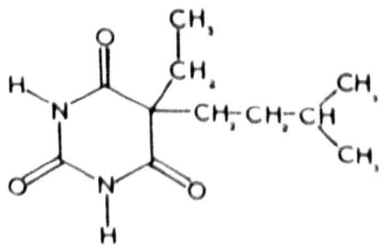
piericidin B $R_1 = H$, $R_2 = CH_3$; monoacetate $R = COCH_3$, $R_2 = CH_3$.

The octahydro derivatives of the above five compounds are those in which the olefinic bonds are saturated.



rotenone

Only the boxed part of the molecule may be chemically modified without affecting the potency of the inhition of NADH dehydrogenase.



amytal

The constitution of NADH dehydrogenase, and the nature of the interactions of inhibitors with it, are complementary subjects which have received a great deal of attention recently.

(i) Inhibitors related to piericidin A

The inhibition of NADH dehydrogenase by piericidin A is very sensitive to chemical modification of the inhibitor. Octahydropiericidin A (figure 7) has been shown to inhibit NADH dehydrogenase at similar concentrations to piericidin A[73,80], but substitution of the phenolic function or loss of the hydrocarbon side chain dissipates the potency of the molecule in this respect. On the other hand, the less potent inhibition of succinate dehydrogenase is not subject to such variations, and is inhibited at μM concentrations by all these compounds.

Rotenone[92], and several rotenoids[87], inhibit NADH dehydrogenase at concentrations almost as low as those of piericidin A. Certain features of the rotenone molecule have been deemed essential for such inhibition[87], (see figure 7). Binding and inhibition studies using radioactive rotenone give very similar results to those already mentioned for piericidin A[91,93] (page 20). One significant difference is the observation that tightly bound rotenone is slowly removed by washing with bovine serum albumen, at a rate much faster than is tightly bound piericidin A[93]. Piericidin A must therefore interact more strongly with NADH dehydrogenase than does rotenone. A number of other minor discrepancies between the behaviour of piericidin A and rotenone have been observed[74].

Certain other compounds have been found to inhibit NADH dehydrogenase at higher concentrations that those of piericidin A and rotenone. Amongst these are amytal (figure 7) and other barbiturates[93,94], certain steroids[95], and a series of analogues of ubiquinone in which the methyl function of quinonoid ring is replaced by a hydroxyl group[96].

Amytal, piericidin A and some rotenoids have been found to decrease the specific binding of radioactive rotenone[91]. Analogously, rotenone and amytal have a similar, but less pronounced effect on the binding of radioactive piericidin A[79,80]. It is concluded that all these compounds can compete for the same binding site, but that piericidin A has the strongest binding affinity.

Of the above compounds, neither the rotenoids nor the barbiturates, both structurally dissimilar to ubiquinone, inhibit succinate dehydrogenase. On the other hand piericidin A and its derivatives, and the series of 2,3-dimethoxy-5-hydroxy-6-alkyl-1,4-benzoquinones, all structurally resembling ubiquinone, do inhibit succinate oxidation. That this inhibition is relieved by the addition of ubiquinone is consistent with the theory that these compounds inhibit succinate dehydrogenase (but not NADH dehydrogenase) by competing with ubiquinone for a common reaction site.

(ii) The constitution of NADH dehydrogenase

There is some controversy over the constitution of the electron transport chain between NADH and ubiquinone. This is chiefly concerned with differences between particulate and other preparations[81-85].

The following scheme is consistent with the majority of current opinions.

NADH $\rightleftharpoons$ Flavoprotein $\rightleftharpoons$ Non-heme iron protein $\rightleftharpoons$ ubiquinone

(F_p) (Fe_p)

Inhibition by piericidin A, rotenone and amytal is proposed, by Singer et al[81-83], to be on the oxygen side of Fep, following EPR studies on their own soluble preparation. It is known that non-heme iron can be detected in its reduced form by a characteristic EPR signal[86]. On the other hand, Hatefi et al[84] have proposed that the inhibition by these compounds is between Fp and Fep. This is based on spectrophotometric studies on their own "complex I", a particulate preparation.

The proximity of none-heme iron to the proposed common binding site for piericidin A, rotenone and amytal is implied by the following observations.

(a) A characteristic EPR signal, attributed to non-heme iron, together with certain optical changes, both remaining after the slow reoxidation of inhibited particles, have recently been put forward as evidence for the involvement of non-heme iron in the binding of piericidin A[88].

(b) Loss of sensitivity towards piercidin A and rotenone (and loss of site 1 phosphorylation) has been observed in mitochondria deficient in iron, from Torulopsis utilis[89]. (Many yeasts normally lack both of these functions[90]). In similar observations with

preparations of Candida utilis starved of iron, no direct correlation is found between loss of sensitivity towards piericidin A, and loss of both the non-heme iron content and the EPR signal attributed to non-heme iron[85]b. It is suggested that the majority of the non-heme iron of NADH dehydrogenase is not concerned with the binding of piericidin A.

Similar circumstantial evidence exists for the involvement of sulphydryl groups in the proposed common binding site.

(a) Sulphydryl reagents (i.e. reagents reacting with sulphydryl groups) have a sunergistic effect when used with piericidin A and rotenone, whereas, 2,3-dimercaptopropanol can actually relieve inhibition of NADH oxidase by piericidin A[74,87].

(b) Sulphydryl reagents added before, but not after, the addition of piericidin A decrease the amount of inhibitor specifically bound to NADH dehydrogenase[82].

NADH dehydrogenase is bound to a membrane, and hence is closely associated with lipids. The possible involvement of hydrophobic interactions with lipids was recognised early as a factor in its inhibition[87].

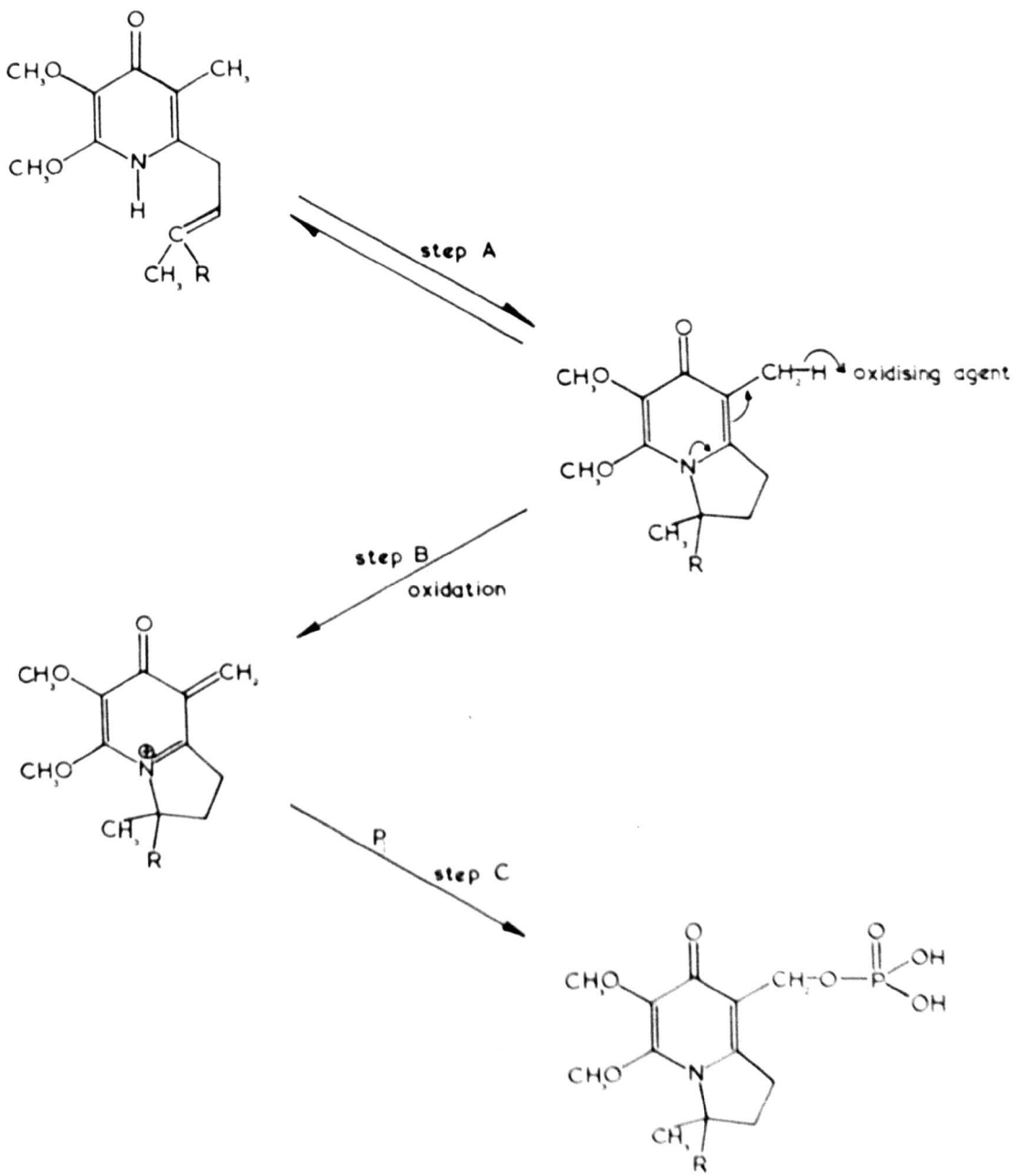
Reagents which release proteins, such as phospholipase[82], release tightly bound inhibitor from the specific binding site of NADH dehydrogenase. Solvent extraction of the mitochondrial lipids also releases tightly bound inhibitors from the mitochondria[70,80,91]. It follows that the interaction between tightly bound ibhibitors and the enzyme is unlikely to involve covalent linkages, such as the formation of a Schiff's base.

In conclusion, proteins, lipids, non-heme iron and sulphydryl groups are all inferred to be in the vicinity of the inhibition/binding site of piericidin A, rotenone and amytal on NADH dehydrogenase. The interactions of these inhibitors do not appear to be covalent, and hence must be of a hydrophobic or ionic nature, or possibly involve ligand interactions with a metal. This latter possibility is one of the few ways in which the inhibitors might be considered to have chemical similarities.

An indirect attempt to reveal more information concerning the interaction of piericidin A in particular with NADH dehydrogenase is one concern of the present work. This is attempted by extending the number of analogues of piericidin A whose effect on the NADH dehydrogenase region of the respiratory chain has been studied, and by investigating the specific binding of radioactive piericidin A to mitochondria.

FIGURE 8

A POSSIBLE REACTION SEQUENCE INVOLVING PIERICIDIN A



CHEMICAL INTRODUCTION

THE POSSIBLE CONTRASTING INVOLVEMENT OF PIERICIDIN A IN A SCHEME ANALOGOUS TO THAT FOR UBIQUINONE

The suggestion that ubiquinone may be involved in oxidative phosphorylation has already been discussed (see figure 6 and accompanying text). Piericidin A may be considered to act in a reaction sequence of parallel nature. This is presented in figure 8. The chief difference would be that nucleophilic attack by phosphate on the methide intermediate would be preceded by an oxidative step instead of followed by one. Furthermore the aralkyl phosphate so formed would be unable to be oxidised in the same sense as ubiquinone, and consequently would be unable to take part in further reactions. Although such a scheme does not account for all the biochemical properties of piericidin A, it has the advantage of accommodating experimental investigation.

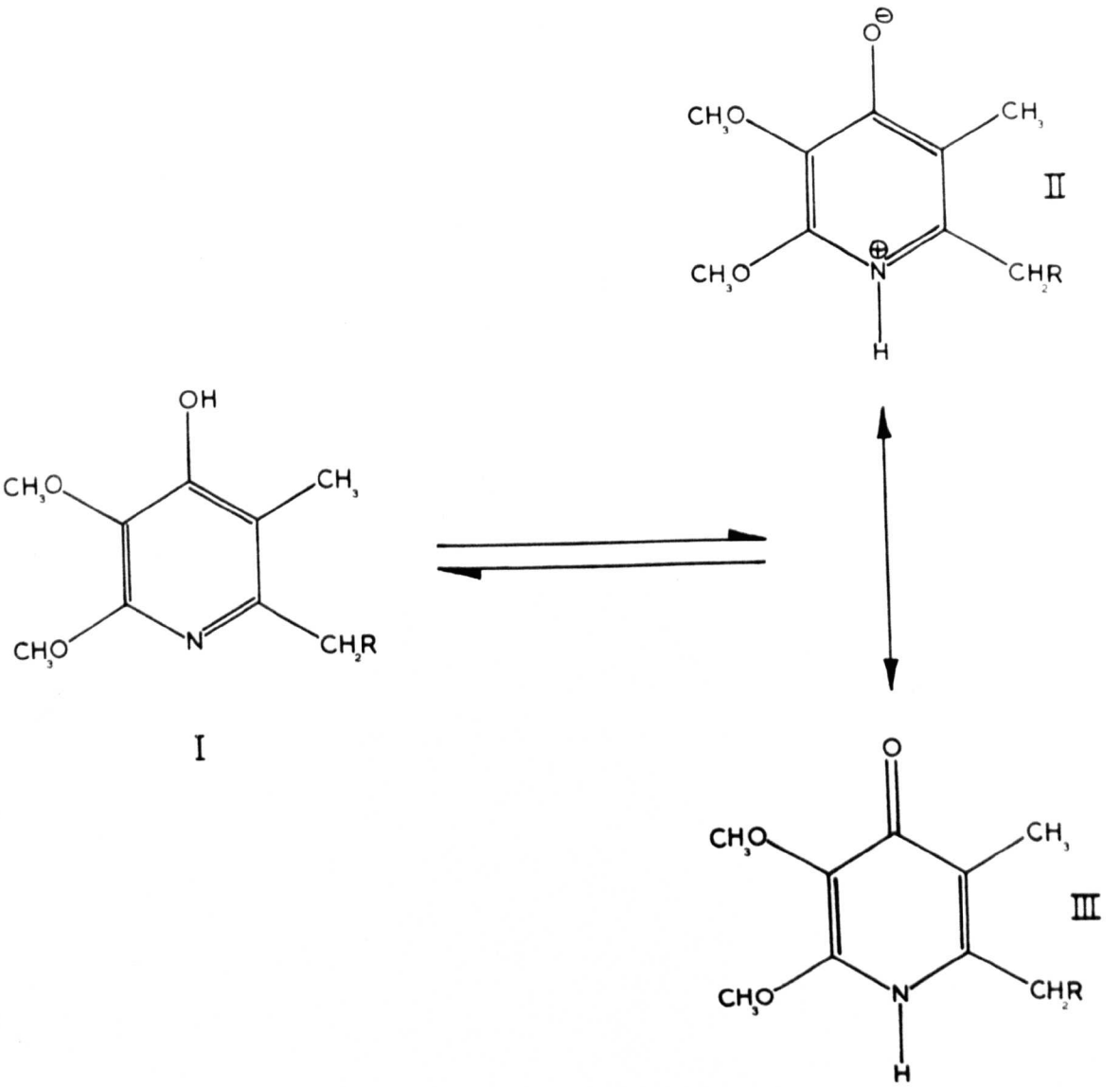
Chemically there are two points at which the above scheme may be tested on model systems. The first is the viability of the cyclisation in step A, and secondly, if this is found, the detection under oxidising conditions of hydrogen loss from the β-methyl function.

A simpler, and perhaps more definitive approach is to investigate the biochemical properties of octahydropiericidin A, in which the proposed cyclisation is not possible. Should there be a considerable change from the behaviour of the parent compound, then the proposed scheme could be responsible for the biochemical action of piericidin A.

On the other hand little or no change would indicate that the mechanism of inhibition is other than that proposed in figure 8.

FIGURE 9

THE TAUTOMERS OF PIERICIDIN A



THE TAUTOMERISM OF PIERICIDIN A

The unique feature of piericidin A is its resemblance to the ubiquinones. It may be an oversimplification to postulate that the aromatic ring of piericidin A is responsible for its ability to inhibit mitochondrial reactions, but the chemistry of such a penta-substituted pyridine certainly deserves due consideration in this context.

Neither the isolation nor properties of a 2,3-dialkyl-5,6-dialkoxy-4-hydroxypyridine have been previously reported. However the properties of such a system can be deduced from a consideration of those of simpler analogues.

As with all 4-hydroxypyridines the molecule may exist in either of two tautomeric forms. These are represented in figure 9. The two forms are clearly distinguishable from each other spectrally, and this aspect will be discussed shortly.

It is possible to calculate the basic pK_a values in aqueous solution of species I and II (figure 9). These will be referred to as pK_{aI} and pK_{aII}. Such calculations agree reasonably well with the measured values of corresponding O-methyl and N-methyl derivatives[97]. From the two pK_a values the relative abundance of the pyridone and pyridinol tautomers may be estimated, in agreement with observations[97].

$$\log_{10} K_T = pK_{aI} - pK_{aII}$$

$$\text{where } K_T = \frac{\text{(Pyridone tautomer)}}{\text{(Pyridinol tautomer)}}$$

From K_T, the tautomeric equilibrium constant, the difference of free energy between the tautomers, ΔG°_T, may in turn be calculated, using the standard free energy relationship:

$$-\Delta G^\circ_T = RT \, Ln K_T$$

Such calculations are made below for the proposed aromatic nucleus of piericidin A.

(a) Calculation of the basic pK_a value of species I, considered as a pyridine

$$pK_{aI} = 5.25 - 5.90 \sum \sigma \quad [98]$$

Values of σ are as quoted in reference 98.

σ_{ortho} (for simple pyridines) - methyl = -0.13

- methoxyl = +0.34

σ_{meta} - methyl = -0.07

- methoxyl = +0.12

σ_{para} - hydroxyl = -0.37

$$\therefore \quad pK_{aI} = 5.25 - 5.90(-0.11)$$

$$= 5.90$$

(b) Calculation of the basic pK_a value of species II, considered as a phenolate ion

$$pK_{aII} = 10.00 - 2.11 \sum \sigma \quad [99]$$

Values of σ originate from the references shown below:

σ_{ortho} – methyl = –0.10
– methoxyl = ZERO
σ_{meta} – methyl = –0.05
– methoxyl = +0.16
σ_{para} $\overset{+}{\rangle}$N–H = +3.19[97]

Calculated from the pK_a values of correspondingly substituted phenols[100],

$\therefore$ $pK_{aII} = 10.00 - 2.11\ (3.20)$

$= 3.25$

(c) Calculation of K_T and ΔG°_T

$\log_{10} K_T = 5.90 - 3.25$

$= 2.65$

$\therefore K_T = 450$

$\therefore -\Delta G^\circ_T = RT \ln 450$

$\therefore \Delta G^\circ_{20^\circ C} = -2 \times 293 \times 5.12$ cals. per mole

$= -3$ Kcals. per mole

Hence in aqueous solution the pyridone tautomer is expected to predominate, and to be of lower free energy.

The only recorded cases of 4-hydroxypyridines existing as pyridinols rather than 1-(H)-pyridones are those in which one or both of the α substituents is a halogen atom[97,101,102]. This is said to be due to a preferential decrease in the basicity of nitrogen compared to oxygen caused by the electron withdrawing substituent in the α position[97,101], and is consistent with the above pK_a evaluations.

The case of 2-hydroxypyridines is more diverse. The pyridinol form is found in some halogenated compounds[101a, 103], presumably for the same reason as that mentioned above. In addition 2-hydroxy-6-methoxypyridine and two comparable 6-alkoxy compounds have been found to exist partly as their pyridinol tautomers[104, 105], although not to an appreciable extent in aqueous solution.

As a general observation, 2-hydroxypyridines would be expected to exist as 1-(H)-pyridones more readily than their 4-substituted counterparts, since the charge separation in the zwitterionic resonance form (see page 33) of the former is less than in that of the latter. This is evident from studies on 2,4-dihydroxypyridine in which the 2-pyridone form was found to predominate, although both 2-and 4-pyridone forms would be possible[106]. Similar results are found in the cases of cyclic keto-lactones and β-ketoesters[107]. Thus the observation that 4-hydroxy-2-methoxypyridine exists principally as a 1-(H)-pyridone[106], whereas 2-hydroxy-6-methoxypyridine can exist partly as a pyridinol[104] is perfectly plausible.

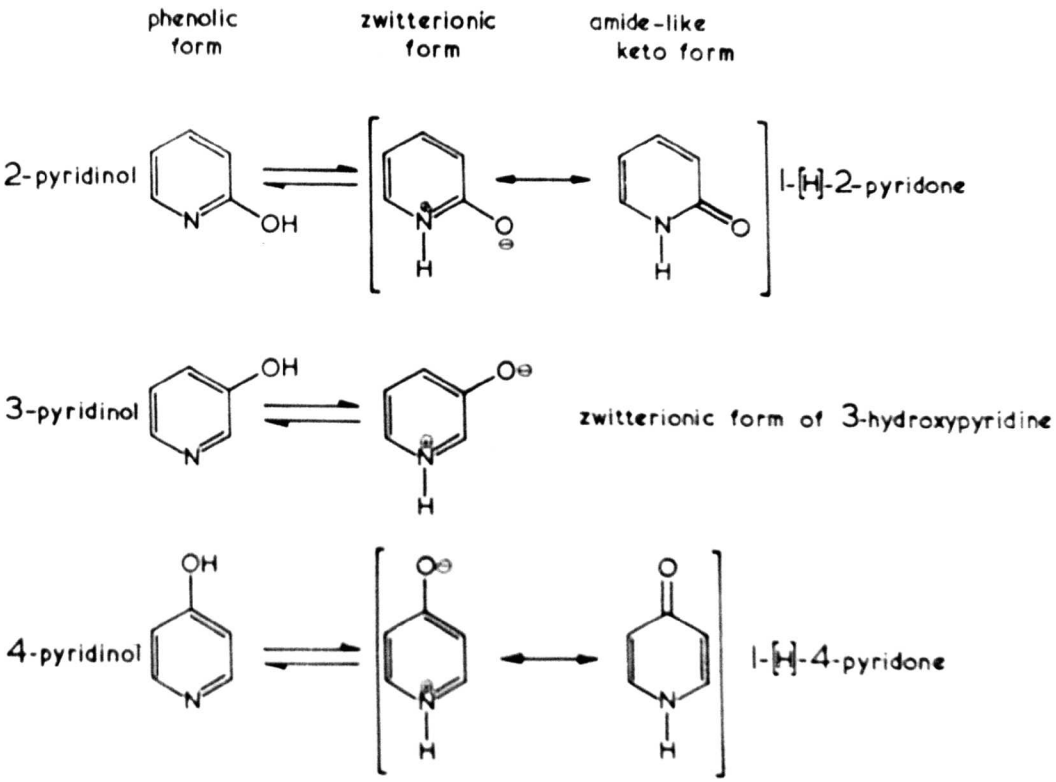
2-Hydroxypyridines[105], 3-hydroxypyridines[108,109] and 4-hydroxypyridines[97,102] which have any appreciable tendency to exist as their pyridinol rather than their pyridone or zwitterionic tautomers are encouraged to do so by solvents of low polarity. This is because the more dipolar pyridone or zwitterionic tautomers are energetically favoured by interactions with dipolar solvents, which are reduced in solvents of low polarity.

An examination of the spectral data of piericidin A in the light of these deductions is preceded by considerations of the spectral and chemical properties of 2-, 3- and 4-hydroxypyridines in their various tautomeric forms.

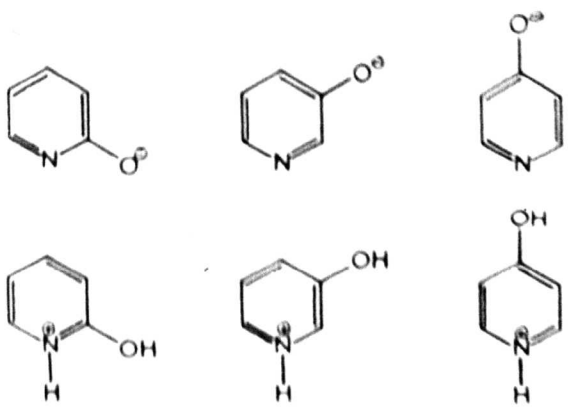
FIGURE 10

TAUTOMERISM IN HYDROXYPYRIDINES

Neutral Molecules



Predominant Forms of Anions and Cations



THE PROPERTIES OF HYDROXYPYRIDINES

All 2-, 3- and 4-hydroxypyridines may exist in two tautomeric forms, representations of which are shown in figure 10. The phenolic form, with a proton on oxygen, is referred to in this thesis as a pyridinol. The other tautomer, with a proton on nitrogen, can be fairly represented by a zwitterionic structure only in the case of a 3-hydroxypyridine. With 2- and 4-hydroxypyridines this tautomer is known as a 1-(H)- pyridone, and is best thought of as intermediate between the zwitterionic and amide-like keto structures shown. Such a structure is of course precluded by the rules of valency from being drawn in the case of a 3-hydroxypyridine.

The chemistry and spectroscopy of the three parent hydroxypyridines is often related in text books to this latter difference[157]. 3-Hydroxypyridine has many typical phenolic properties, whereas the other two isomers are found to exist predominantly as 1-(H)-pyridones and behave differently. Many more highly substituted hydroxypyridines reflect the chemistry of these parent compounds, but the existence of a small number of 2- and 4-hydroxypyridines in which the phenolic pyridinol tautomer predominates (see pages 30-31) necessitates the subdivision of generalisations under these headings.

Despite some initial doubt[110], the anions and cations of all three hydroxypyridines are now believed to exist substantially in Kekulé forms as represented in figure 10[110-114].

The ultra-violet absorption spectra of hydroxypyridines in aqueous solution

(i) 2-hydroxypyridines[102,104-106,111,115-117]

(a) 1-(H)-2-pyridones

2-hydroxypyridine[116]	λ max (nm.)	ϵ max
Neutral molecule	293	5,890
Anion	291	5,070
Cation	277	6,950

Many substituted 2-hydroxypyridines exist predominantly as 1-(H)-2-pyridones and have U.V. spectra reminiscent of 2-hydroxypyridine itself[117]. The additional substituents frequently produce a bathochromic shift of the absorption maximum of the neutral molecule compared to that of the parent compound. The absorption maximum in basic solution is usually of a slightly lower wavelength and extinction coeffieient than that in neutral solution, that in acidic solution being usually of lower wavelength but higher extinction coefficient.

(b) 2-pyridinols

Tetrachloro-2-hydroxypyridine[102]	λmax (nm.)	ϵmax
Neutral molecule	305	5,400
	330	4,500
Anion	244	7,000
	321	6,400
Cation	327	6,900

Too few examples of 2-pyridinols are known to make generalisations concerning their UV. spectra, but the spectra of 2-alkoxypyridines may be borne in mind[102,111,116,117]. In acidic solution these often have absorption maxima of higher wavelength and extinction coefficient that those in neutral solution.

(ii) 3-Hydroxypyridines[108,109,115,116,118]

3-hydroxypyridine[116]	λ max (nm)	ε max
Neutral molecule (pyridinol)	278	2,320 (variable)
(zwitterion)	315	3,060 (variable)
Anion	298	4,960
Cation	283	5,840

Additional substituents frequently produce bathochromic shifts of the absorption maxima of the neutral species compared to those of the parent compound. Two neutral species, one a pyridinol, the other a zwitterion, are often detected, the latter having an absorption maximum at higher wavelength. The extinction coefficient of each will vary since it is related to the proportion of the particular species present, which in turn depends on the polarity of the solvent. The absorption maxima in acidic and basic solutions are usually of greater wavelength than those of the neutral pyridinol species. The absorption maximum in acidic solution also commonly has a greater extinction coefficient than the combined values of those of the two neutral species.

(iii) 4-hydroxypyridines

(a) 1-(H)-4-pyridones[2b,97,102,106,115,116]

4-hydroxypyridine[116]	λ max (nm.)	ε max
Neutral molecule	253	14,800
Anion	239	14,150
	260 (shoulder)	(2,200)
Cation	234	9,800

Many substituted 4-hydroxypyridines exist predominantly as 1-(H)-4-pyridones and have UV spectra reminiscent of 4-hydroxypyridine itself. Owing to their considerable dipolar nature these molecules have extinction coefficients greater than other comparable pyridines. Additional substituents usually produce a bathochromic shift of the absorption maximum in neutral solution compared to that of the parent compound. In acidic and basic solutions a hypsochromic shift of the absorption maximum compared to that in neutral solution is commonly, but not exclusively, found.

(b) 4-pyridinols[97,101b,102]

Too few examples of 4-pyridinols are known to enable generalisations concerning their UV spectra to be made. These examples are shown below.

Tetrafluoro-4-hydroxypyridine	max (nm.)	max
Neutral molecule	243	2,270
	255	1,790
Anion	233	11,450

Tetrachloro-3-hydroxypyridine	λ max (nm.)	ε max
Neutral molecule	235	7,900
	272	2,200
Anion	253	5,900
	278	3,000
Cation	253	4,200
	287	5,000

2,6-dichloro-4-hydroxypyridine	λ max (nm.)	ε max
Neutral molecule	260	1,900
Anion	244	10,400
Cation	240	7,400
	262	6,800

The infra-red absorption spectra of hydroxypyridines

Pyridinols and 1-(H)-pyridones can be clearly distinguished from each other by their IR spectra. In solution, 3-hydroxypyridines[121], and such 2-hydroxypyridines[102] and 4-hydroxypyridines[97,101,102] that exist as pyridinols exhibit a strong sharp absorption in the region of 3,500-3,600 cm^{-1}, typical of free O-H stretching vibration. This is not found with 1-(H)-pyridones, which additionally absorb in regions where pyridinols do not.[103,117,121,122] 1-(H)-2-Pyridones absorb strongly within the range 1635-1700 cm^{-1}, and also less strongly, often with more than one peak and in a very broad manner within the range 2700-3420 cm^{-1}. 1-(H)-4-Pyridones[97,121,122c], similarly, absorb with the ranges 1620-1685 cm^{-1} and 2500-3445 cm^{-1}. The higher wavenumber absorption in each case is attributed to N-H stretching vibrations. The lower wavenumber absorption has been

attributed to C=O stretching vibrations[121,122a], and to vibrations within the pyridine ring[119,120]. Studies using compounds enriched with ^{18}O, ^{15}N or ^{2}H [122a,b,c], lead to the conclusion[122b,c] that this absorption is due to a vibration of a mixed nature, since, although its frequency is reduced in compounds containing ^{18}O in place of ^{16}O, this reduction is less than would be expected from the relationship commonly used to estimate the stretching frequency, ν_{St}, of a chemical bond.

$$\nu_{St} = \frac{1}{2\pi c} \sqrt{k\left[\frac{1}{M_1} + \frac{1}{M_2}\right]}$$

(Where c is the velocity of light, k the force constant of the bond and M_1 and M_2 the masses of the two bonded atoms).

Moreover the frequency of absorption is effected by changing the isotope of nitrogen or hydrogen present, leading to the same conclusion.

The ^{1}H nuclear magnetic resonance spectra of hydroxypyridines

^{1}H NMR studies on 2-hydroxypyridine[123] and 4-hydroxypyridine[124], together with the corresponding 1-methylpyridones, have established differences in the chemical shifts of their nuclear protons compared to those of the corresponding methoxy compounds. The resonance frequencies of the protons of the hydroxypyridines (and 1-methylpyridones) are found at higher τ values, implying a reduced aromatic ring-current effect in these molecules. The degree of this increase in τ value depends on the position of substitution of the nuclear proton, in the following manner:

2-hydroxypyridine: 6>5>4>3

4-hydroxypyridine: 2 and 6>3 and 5

The general effect of additional substituents on the chemical shifts of existing ones, particularly protons, in pyridines may be summarised by saying that electron-withdrawing substituents deshield existing substituents (lowering their τ values), whilst electron-releasing substituents have the opposite effect[97,123,128].

Studies concerning changes of coupling constants with substituent[123], or on protonation of nitrogen[123,125], have been made, but these are hardly relevent to a penta-substituted pyridine such as that present in piericidin A. As mentioned earlier (page 33), the cations of 2- and 4-hydroxypyridine have been confirmed by ^{1}H NMR spectroscopy to exist substantially in Kekulé forms[112,113,124,126]. The resonance frequencies of protonic substituents are usually shifted to lower τ values on the protonation of nitrogen, this again being an effect of the withdrawal of electrons from the aromatic ring. The effect is greater in the β and γ positions of the pyridine ring than in the α positions[123,124,127].

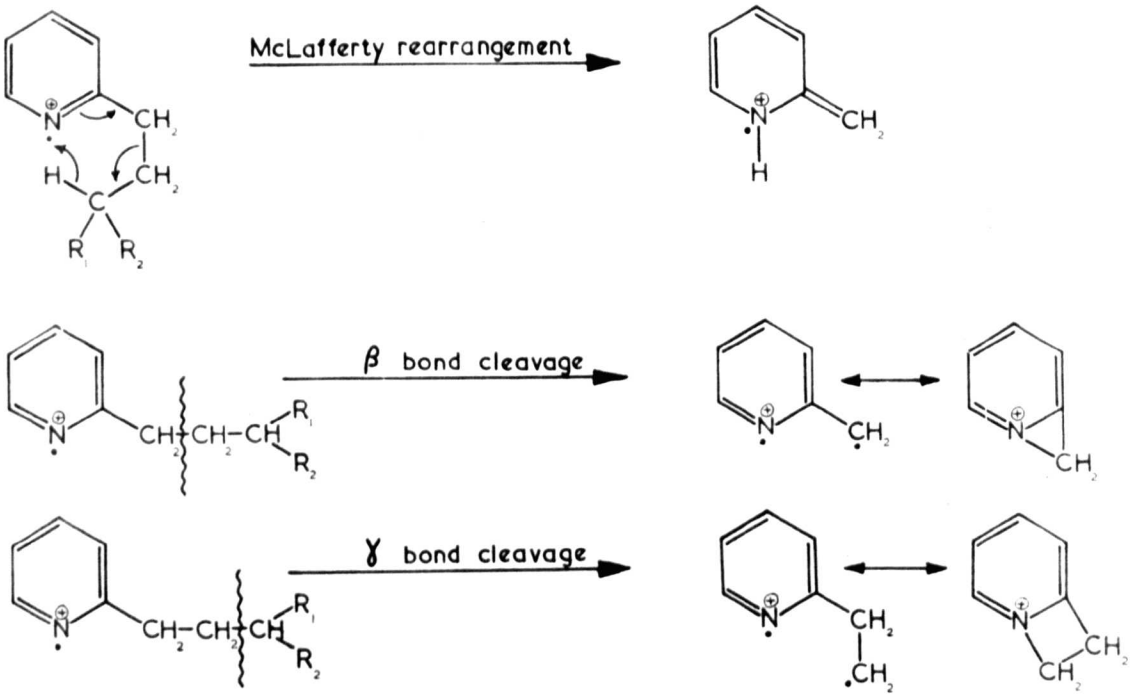
As a final observation it might be expected, from a consideration of the NMR spectrum of pyridine itself[129], that the protons of a particular substituent in an α position in a pyridine would resonate at a lower τ value than those of the same substituent in a β or γ position provided that the remainder of the molecule was comparable.

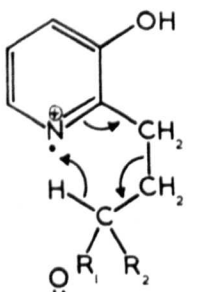
<u>The mass spectrometry of hydroxypyridines</u>[130]

The mass spectra of 2,-3- and 4-hydroxypyridine have been published[131].

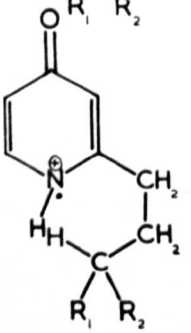
FIGURE 11

FRAGMENTATIONS IN THE MASS SPECTROMETER





McLafferty rearrangement likely



McLafferty rearrangement unlikely

Molecular ions of 2-hydroxypyridine decompose principally by eliminating carbon monoxide, whereas those of 3- and 4-hydroxypyridine also eliminate hydrogen cyanide with comparable frequency. The principal fragments of primary decomposition of a large number of 2-hydroxypyridines and 1-methyl-2-pyridones appear to be formed by the elimination of either carbon monoxide or a formyl radical[132-135]. 1-methyl-4-pyridone also decomposes by elininating carbon monoxide[136]. Other examples of simple 3- and 4-oxygenated pyridines have not been reported.

A study of the mass spectra of the seven hydroxyquinolines and their methoxy and 1-methyl derivatives, relevent to the analogous pyridinic examples, has been made[137]. The major primary fragmentation process of all the hydroxyquinolines and of the two 1-methyl derivatives is the elimination of carbon monoxide. The methoxy derivatives eliminate either formaldehyde or a neutral species of mass 43, identified as an acetyl radical, in their major initial fragmentation.

One further piece of information of particular relevence to the mass spectra of the octahydropiericidins is included here, although it does not concern hydroxypyridines in general. Any pyridine, substituted in an α position with a saturated carbon chain of at least three carbon atoms, possessing a hydrogen atom on the third carbon atom, will preferentially fragment <u>via</u> a McLafferty rearrangement in the mass spectrometer[138], rather than by β or γ bond cleavage (see figure 11). A molecule with the same substituent in a β or γ ring position will fragment principally by β or γ bond cleavage, and not <u>via</u> a McLafferty rearrangement.

The ionisation constants of hydroxypyridines

Hydroxypyridines are both weak acids and weak bases. The pK_a values of the three simplest examples have been measured[139].

	Basic pK_a	Acidic pK_a
2-hydroxypyridine	0.74	11.62
3-hydroxypyridine	4.86	8.72
4-hydroxypyridine	3.27	11.09

In addition the basic and acidic pK_a values of each extreme tautomeric form have been estimated[140].

	Basic pK_a	Acidic pK_a
2-pyridinol	3.71	8.66
3-pyridinol	5.22	8.36
4-pyridinol	6.56	7.80
1-(H)-2-pyridone	0.75	11.62
zwitterion of 3-hydroxypyridine	5.12	8.46
1-(H)-4-pyridone	3.27	11.09

It is clear that 2- and 4-hydroxypyridines existing predominantly as 1-(H)-pyridones can be expected to be weakly phenolic and very weakly basic. Most 3-hydroxypyridines can be expected to be slightly more acidic than a corresponding phenol and at the same time approach the basic strength of pyridine. However any 2- or 4- hydroxypyridines existing predominantly as pyridinols are likely to be quite strong acids. For example tetrachloro-4-pyridinol has an <u>acidic</u> pK_a value as low as 2.0[102], and that of tetrafluoro-4-pyridinol is 3.21[101b]. Both compounds are also <u>very</u> weak bases.

Chemical differences between hydroxypyridines of relevence to piericidin A

(i) Spray tests

The possibility of a facile colour test, applicable as a spray, to provide some distinction between 2-, 3- and 4-hydroxypyridines has not escaped the imagination of chemists. However, neither ferric-chloride solution[141,142], nor the Folin-Denis reagent[141,143,144], have proved foolproof in this respect.

(ii) Acyl derivatives[147]

2-, 3- and 4-Hydroxypyridines are all acylated on oxygen rather than nitrogen. 3-Acetoxypyridine behaves most like a phenyl acetate, but 2- and 4-acetoxypyridines are unstable and are easily hydrolysed, and need to be prepared under anhydrous conditions[145].

(iii) Alkyl derivatives

3-Hydroxypyridines, which in many respects behave as typical phenols, may be alkylated on nitrogen by methyl iodide, dimethyl sulphate and diazomethane. However diazomethane will methylate 3-hydroxypyridine in homogeneous solution to give 3-methoxypyridine. 2- and 4-hydroxypyridine are alkylated on both oxygen and nitrogen, and it is quite usual to isolate both products. Basic conditions favour alkylation on nitrogen. In connection with the preparation of "O-methyloctahydropiericidin A" [2a], it should be noted that 4-hydroxy-pyridine is methylated exclusively on nitrogen in the presence of methyl iodide and silver oxide[146].

A brief summary of this section is included below.

(i) 1-(H)-2- and 1-(H)-4-Pyridones, and 3-hydroxypyridines behave

predictably and differently in their absorption of ultra-violet radiation in neutral, basic and acidic solutions. No such generalisation is evident in the case of 2- and 4-pyridinols.

(ii) It is possible to distinguish between 1-(H)-2-and 1-(H)-4-pyridones, on the one hand, and 2-,3- and 4-pyridinols on the other, by infra-red spectroscopy

(iii) It may be possible to distinguish closely related poly-substituted hydroxypyridines by ^{1}H NMR spectroscopy, particularly with reference to the location of substituents. Too few examples are reported to enable a more precise statement to be made at this stage.

(iv) Mass spectrometry is unlikely to distinguish between isomeric poly substituted hydroxypyridines.

(v) There are noticeable differences between the pK_a values of 1-(H)-pyridones and pyridinols. Pyridinols are usually more acidic.

(vi) 2- and 4-Acetoxypyridines are much less stable and more easily hydrolysised than are 3-acetoxypyridines. All three hydroxypyridines are acylated on oxygen, but may be alkylated on either oxygen or nitrogen. They cannot be reliably distinguished by a simple spray test.

THE PROPERTIES OF PIERICIDIN A COMPARED WITH THOSE OF OTHER HYDROXYPYRIDINES

(i) Ultra-violet absorption spectrum[2a]

The UV spectral absorptions of octahydropiericidin A are quoted, since in this compound they can only be attributed to the pyridine nucleus, whereas in the case of piericidin A dienic absorptions may interfere. The spectra were recorded in aqueous methanolic solution.

Octahydropiericidin A	λ max (nm.)	ϵ max
Neutral molecule	267	5,300
Anion	No peak above 230	Approx.3,000 at 270 nm.
Cation	275	8,500

The bathochromic shift in acidic solution is typical of a 3-hydroxypyridine, but not of 1-(H)-2-or 1-(H)-4-pyridone (see pages 34-37). The behaviour in basic solution is atypical of any hydroxypyridine.

(ii) Infra-red absorption spectrum[2a]

No significant absorption of radiation is found with piericidin A between 1630 and 1700 cm^{-1}. The weak absorption of piericidin A occurring at 1620 cms^{-1}, and not found in octahydropiericidin A, is attributed to olefinic C=C stretching vibrations. On the other hand absorptions at 3470 cm^{-1} and 3560 cm^{-1} typical of hydrogen bonded and "free" O-H stretching vibrations are found. It is concluded that piericidin A is a pyridinol and not a 1-(H)-pyridone (see pages 37-38).

(iii) ^{1}H NMR spectra of piericidin A and octahydropiericidin A are consistent with the proposed substituents of the pyridine ring of the two compounds. However no conclusion as to the location of these

substituents in particular positions in the ring can be made from the spectra, without recourse to the spectra of appropriate analogues. Insufficient of these have been reported. (See pages 38-39).

(iv) Mass Spectrum

Neither the mass spectrum of piericidin A nor that of octahydropiericidin A have been reported. The mass spectra of three acetylated derivatives of octahydropiericidin A are however available[2e]. They are consistent with the proposed structure of octahydropiericidin A, and indicate that the side-chain is substituted in an α position, since, in each case, a McLafferty rearrangement is observed (see page 40).

It seems reasonable to suppose that, in the case of a 1-(H)-4-pyridone such as may exist in piericidin A, a McLafferty rearrangement would be less facile on electronic grounds than in the case of a 3- or 4-pyridinol (see figure 11). Such a supposition needs verification, but nevertheless, since the rearrangement is demonstrable in the octahydropiericidins, this may prove to be evidence for the presence of a pyridinol (see pages 114-116).

No conclusion concerning the location of other ring substituents can be made from the mass spectra.

(v) pK_a value

The acidic pK_a value of piericidin A has been measured as 10 in 50% methanolic solution[2a]. Most 2- and 4-pyridinols are more acidic than this (see page 41). 1-(H)-2- and 1-(H)-4-pyridones, having two alkyl and two methoxyl substituents, would be expected to be slightly

less acidic than the unsubstituted compounds (see page 41), and would probably have acidic pK_a values of about 12. 3-hydroxypyridines, having two alkyl and two methoxyl substituents, would likewise be expected to be slightly less acidic than the parent compound, and might very well have acidic pK_a values of about 9.5. This corresponds to the value observed in the case of piericidin A, suggesting that this compound is a 3-hydroxypyridine.

(vi) Chemical observations

The unquestioned stability of piericidin A diacetate and of octahydropiericidin A mono- and di-acetates[2a], implies that these compounds are 3-acetoxypyridines rather than 2- or 4-acetoxypyridines (see page 42).

The reported "O-methylation" of octahydropiericidin A with methyl iodide and silver-oxide[2a] is inconsistent with the behaviour of 4-hydroxypyridine which is methylated on nitrogen under these conditions[146]. Furthermore no change of the UV spectrum of "O-methyloctahydropiericidin A" is observed in acidic and neutral solutions[2a]. This is unusual for a methoxypyridine[116], and suggests that the compound may be a dimethylated quaternary salt.

In conclusion, it can be said that under conditions where distinctions have been observed between the behaviour of pyridinols and 1-(H)-pyridones, piericidin A behaves as a pyridinol and not as a 1-(H)-pyridone. It was concluded from an earlier calculation (page 30) that a 2,3-dialkyl-5,6-dimethoxy-4-hydroxypyridine, such as is proposed

for the structure of piericidin A[2,3], should exist as a 1-(H)-4-pyridone and not as a 4-pyridinol.

This dichotomy has two solutions:

(i) Piericidin A is a 3-hydroxypyridine and not a 4-hydroxypyridine.

(ii) The proposed 2,3-dialkyl-5,6-dimethoxy-4-hydroxypyridine moiety of piericidin A[2,3], has the unique and unexpected property of existing as a pyridinol and not as a 1-(H)-pyridone.

It is clearly of interest to consider the evidence put forward by Takahashi *et al*[2,3] for the proposed substitution of the pyridine ring of piericidin A.

EVIDENCE CONCERNING THE STRUCTURE OF THE PYRIDINE RING OF PIERICIDIN A

The presence of a pyridine ring in piericidin A bearing the following substituents (methyl, two methoxyl, hydroxyl and a methylene group bearing a $C_{16}H_{25}O$ side chain) is consistent with all the reported UV, IR, ^1H NMR and mass spectral observations[2a,2e,3]. Zeisel methoxyl determinations, spray tests and an acidic pK_a value of 10, together with the isolation of a phenolic acetate, are also in line with such a structure[2a].

The proposed arrangement of substituents in the pyridine ring[2,3] is shown in figure 1, and has been deduced from two lines of investigation:

(i) The identification of fragments arising from the ozonolysis of octahydropiericidin A and its derivatives, attributed to the pyridine ring of these compounds.

(ii) Comparisons of the UV spectra of piericidin A, and of octahydropiericidin A, with those of two similar synthetic pyridines.

(i) Fragments arising from ozonolysis

(a) The isolation and identification of $CH_3O.CO.NH.CO.CH_2.R$ (where $R=C_{16}H_{32}(OCO.CH_3)$) from the ozonolysis of octahydropiericidin A diacetate[2b] is convincing evidence for the presence of the two proposed α substituents of the pyridine ring (a methoxyl group and a long hydrocarbon side-chain). As already mentioned (page 45), mass spectral data confirms the presence of the long hydrocarbon side-chain in an α position in the ring[2e].

(b) The isolation of the 2,4-dinitrophenylhydrazone of methyl pyruvate after the ozonolysis of "O-methyloctahydropiericidin A", but not after the ozonolysis of octahydropiericidin A, is cited as evidence for the adjacency of hydroxyl and methyl functions in the pyridine ring of the parent compound. Identification of the derivative was by mixed melting-point and IR spectroscopy. The recorded melting-point is 140-143°C[2b]. Literature values for the melting point of the 2,4-DNP of methyl pyruvate are inconsistent, being 142-144°C[148], 186.5-187.5°C[149] and 184-185°C[150]. In addition the 2,4-DNP derivatives of α-keto esters[150], and α-keto acids[151], have been shown to exist in two forms. This ambiguity throws doubt on the identity of the compound isolated by Takahashi[2b]. Even if this compound has been identified correctly, its isolation only proves the adjacency of methoxyl and methyl functions in "O-methyloctahydropiericidin A". Failure to isolate the same compound from the phenolic parent compound is not ipso facto evidence for the adjacency of hydroxyl and methyl functions in this molecule and piericidin A. The deception of this evidence appears to have passed unnoticed.

(ii) Ultra-violet spectral comparisons

Comparisons of the UV spectra of piericidin A, and of octahydropiericidin A, with those of compounds VII and VIII (figure 12), both derived from kojic acid, are furnished as conclusive proof of the proposed structure of the pyridine ring of piericidin A[2b, 2d].

Octahydropiericidin A	λ max (nm.)	ϵ max
Neutral Molecule	267	5,300
Anion	No peak above 230	Approx.3,000 at 270
Cation	275	8,500

Compound VII	λ max (nm.)	ε max
Neutral molecule	274	11,500
Anion	250	8,500
	270 (shoulder)	5,300
Cation	244	5,150
	270	5,300

pH ?

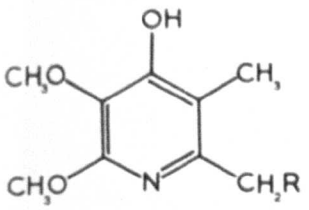
Compound VIII	λ max (nm.)	ε max
Neutral molecule	262	3,700
Anion	241	8,300
Cation	268	7,200

The identity of compound VII is firmly established[152], however its UV spectra are not very similar to those of octahydropiericidin A.

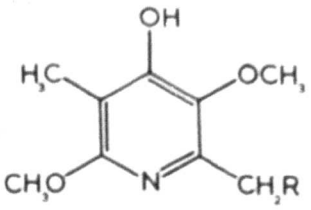
The UV spectra of compound VIII, synthesised by Takahashi[2d], resemble those of octahydropiericidin much more closely. On the other hand their dissimilarity from those of compound VII and other 1-(H)-4-pyridones (see page 36) is surprising. The only other spectral data of compound VIII quoted are some IR absorptions; namely: ν_{max}^{nujol} 3,200 1,620, 1,600, 1,125 cm^{-1} [2d]. The first two of these figures suggest that the compound exists as a 1-(H)-pyridone (see page 37), and certainly no equivalent absorptions are found in the IR spectrum of octahydropiericidin A, which appears to exist as a pyridinol (see page 44). Bearing in mind this difference, and the fact that no other spectral comparisons are made between the above-mentioned compounds, the similarity of the UV spectra of octahydropiericidin A and of compound VIII cannot be regarded as irrefutable evidence for the proposed location of

FIGURE 12

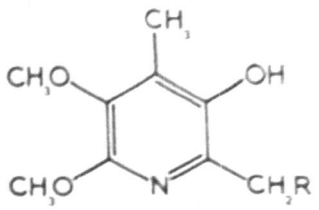
THE SIX POSSIBLE ISOMERIC STRUCTURES OF THE PYRIDINE NUCLEUS OF PIERICIDIN A



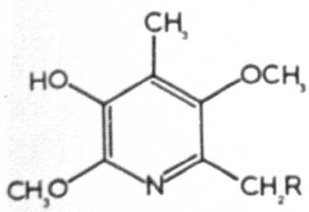
I



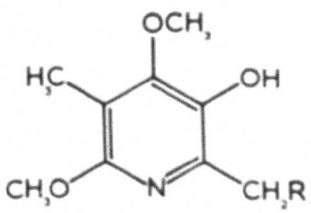
II



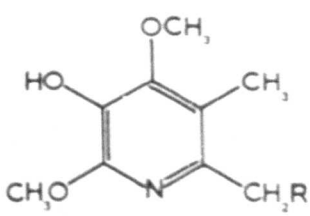
III



IV

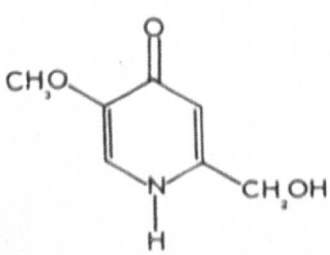


V

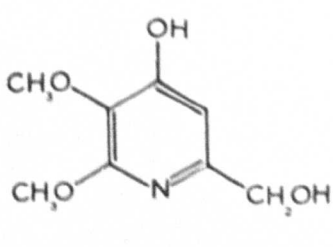


VI

TWO DERIVATIVES OF KOJIC ACID



VII



VIII

substituents on the pyridine ring of octahydropiericidin A (and of piericidin A).

It is a contention of this thesis that the published structure of piericidin A[2,3] may be incorrect. The reviewed evidence allows that any one of five other isomeric structures of the pyridine nucleus could also be that of piericidin A. The six possible isomeric structures are shown in figure 12 (I-VI). Of these compounds I and II would be expected to exist as 1-(H)-4-pyridones (see pages 28-30), and the remainder as pyridinols. Since piericidin A behaves as a pyridinol (see page 46), it is likely that the correct structure is one of these latter four isomers.

One of the aims of the present work is the identification of the correct structure of the pyridine nucleus of piericidin A, by extension of both the number and the kind of comparisons between it an appropriate pyridinic analogues. Some of these will need to be specially synthesised, since they have not been previously reported.

THE SIDE-CHAIN OF PIERICIDIN A

A simple experiment designed to confirm the location of olefinic bonds in the side-chain of piericidin A has been undertaken as part of the present work. The mass spectra of octahydropiericidin A derivatives contain peaks attributable to ions formed by the cleavage of sequential C-C bonds of the side chain[2e]. In this way the carbon framework of the side-chain is reliably revealed, with the exception of the location of the olefinic bonds of piericidin A. The mass spectrum of ($^{2}H_{8}$)-octahydropiericidin A should reveal these latter features on comparison of the two spectra. The synthesis of such a compound by catalytic ($^{2}_{H}$)-hydrogenation of piericidin A should be easy.

The above experiment is prompted by the unusual UV absorptions attributed to the diene system of piericidin A[2a], namely the maxima at 232 nm (ϵ_{max} = 39,500) and 239 nm. (ϵ_{max} = 40,500). A single trans-trans diene, such as is proposed[2c], would be expected to have a single absorption maximum in this region, having an extinction coefficient not exceeding a limit of 30,000[153]. The doublet, of extinction coefficient 40,000, implies the presence of two independent dienic systems, as was once proposed[2a].

The structure of piericidin B[154a,155], in which the alcoholic function of piericidin A is methylated, and a detailed investigation of the stereochemistry of the side-chain of piericidin A[154b,155], have been published.

The incorporation of propionate and acetate, rather than of mevalonate, during the biosynthesis of piericidin A, and of piericidin B, has been proved, although the origin of the nitrogen atom of the pyridine ring of these compounds remains obscure [156].

RESULTS AND DISCUSSION

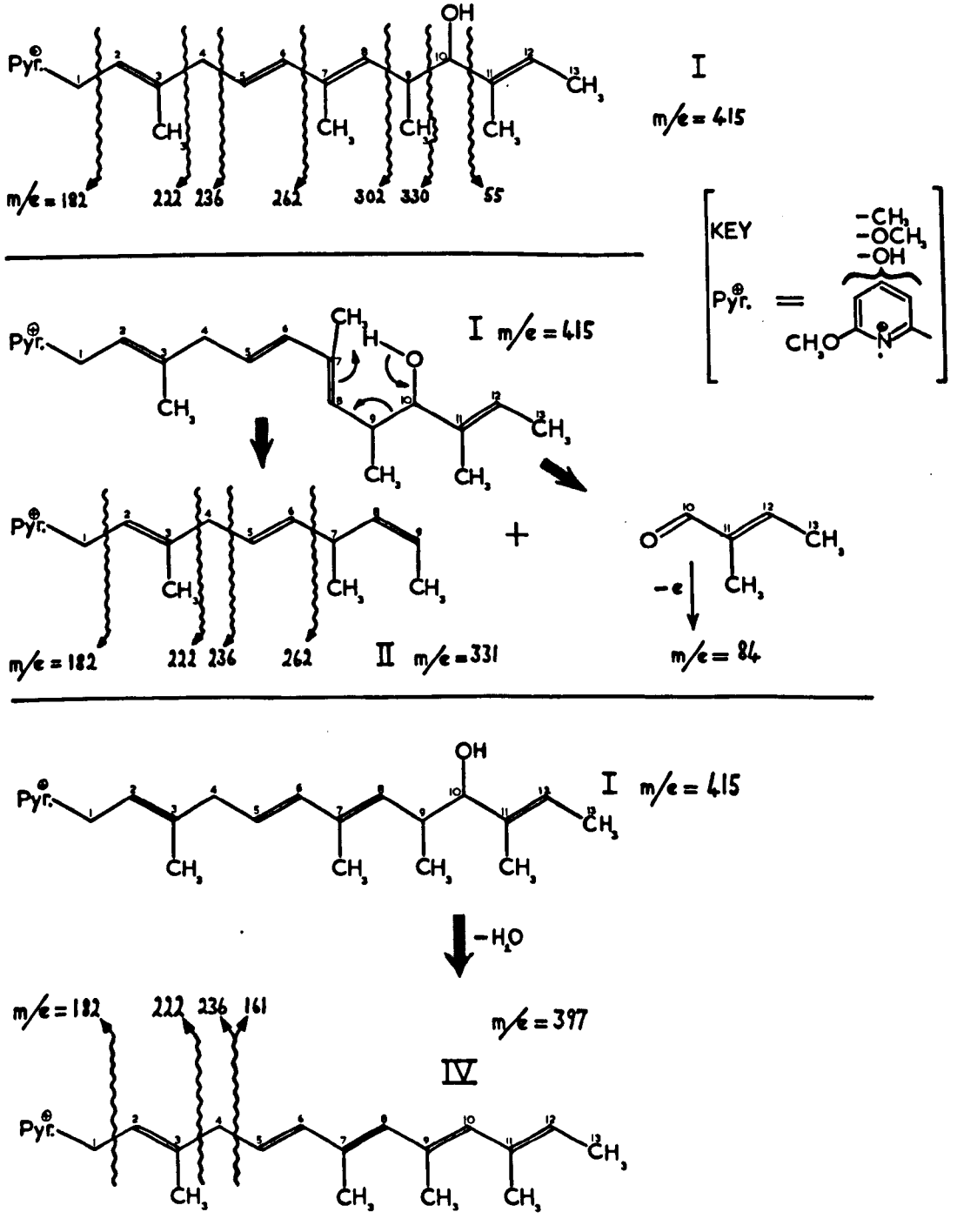
METABOLITES OF STREPTOMYCES MOBARAENSIS AND THEIR DERIVATIVES

Piericidin A and piericidin B were isolated from the mycelia of Streptomyces mobaraensis in the manner described by Takahashi[1] et al.[1] The fungus was grown on two media, one very rich[1], and the other relatively simple[156] (see pages 130-1). The yield of piericidin A was lower than that reported by Takahashi[1] in both cases, the simpler medium giving a slightly higher yield of 17 mg./l. of medium. The yield of piericidin B was 3.6 mg./l. of medium in both cases. During the isolation procedure, relatively large quantities of fatty substances were recovered from the early column eluents, but were not investigated further. Two other metabolites, both crystalline and referred to as C and D, were isolated from fermentations using the simple medium, but not from those using the rich medium. The yields of these, neither of which have been previously reported, were approximately 2 and 1 mg./l. of medium respectively. The IR (thin film), UV and ^{1}H NMR spectrum of piericidin A and piericidin B were similar to those reported by Takahashi[1,2a 154a] (see pages 131-134 and figure 35). In addition the IR spectrum of piericidin A in solution revealed a strong and sharp absorption at 3505 cm^{-1} typical of a pyridinol, and no absorptions attributable to a 1-(H)-pyridone (See pages 37-38, 132). ^{1}H NMR spectra of piericidin A and piericidin B after reaction with trichloroacetylisocyanate were also recorded and are discussed later (pages 108-109 and figure 36.)

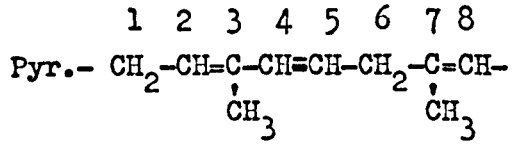
Piericidin A has a very low vapour pressure at room temperature, however a mass spectrum was obtained at high gain and an ionisation chamber temperature of 200°C (see figure 40 of the mass spectral

FIGURE 13

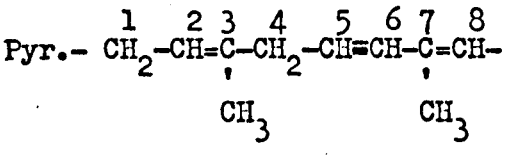
THE MASS SPECTRAL BEHAVIOUR OF PIERICIDIN A



appendix). A molecular ion at m/e = 415 was detected, with a much more prominent ion at m/e = 397, presumably formed by the loss of water from the parent. Accurate mass measurements of six other prominent peaks of the spectrum were made (see page 133). As a result a scheme for the behaviour of piericidin A is postulated in figure 13. In order to account for the intense ions at m/e = 161 and m/e = 236, a change in the location of one olefinic bond in the side chain, from that of the published structure[2e], is necessary.



becomes

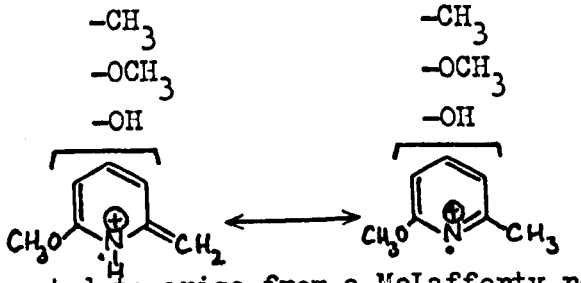


On inspection the two structures are seen to be very similar, and would not be expected to have markedly different UV, IR and ^{1}H NMR spectra. Indeed, the possibility that either structure may be correct has already been recognised[2e]. It is now proposed that the lower structure is correct on the basis of the mass spectrum of piericidin A.

The molecular ion, I (figure 13), may decompose in a variety of ways depending, presumably, on the orientation of the molecule and on the location of positive charge. The elimination of either water (I⟶IV) or of 2-methylbutanal (I⟶II) gives rise to ions at m/e = 397 and 331 respectively. 2-methylbutanal itself also features prominently as an ion at m/e = 84. Fission of ion IV between C_4 and C_5 produces prominent ions at m/e = 236 and m/e = 161. The stability of this latter hydrocarbon fragment, borne out by the

intensity of the ion, is to be expected from a highly conjugated system such as the one proposed[158]. Fission of species I, II or IV between C_1 and C_2, C_3 and C_4 and C_4 and C_5 can be considered to be the origin of ions at m/e = 182, 222 and 236 respectively. Ions at m/e = 330 and 55 can result from fission of the molecular ion (I) on either side of C_{10}, the carbon atom bearing the alcoholic function. Less intense ions at m/e = 262 and 302 correspond to fission principally of I between C_6 and C_7, and C_8 and C_9 respectively. An ion of similarly low intensity at m/e = 282 may originate from IV by the loss of an unspecified methyl radical.

The intense peak at m/e = 183 corresponding to



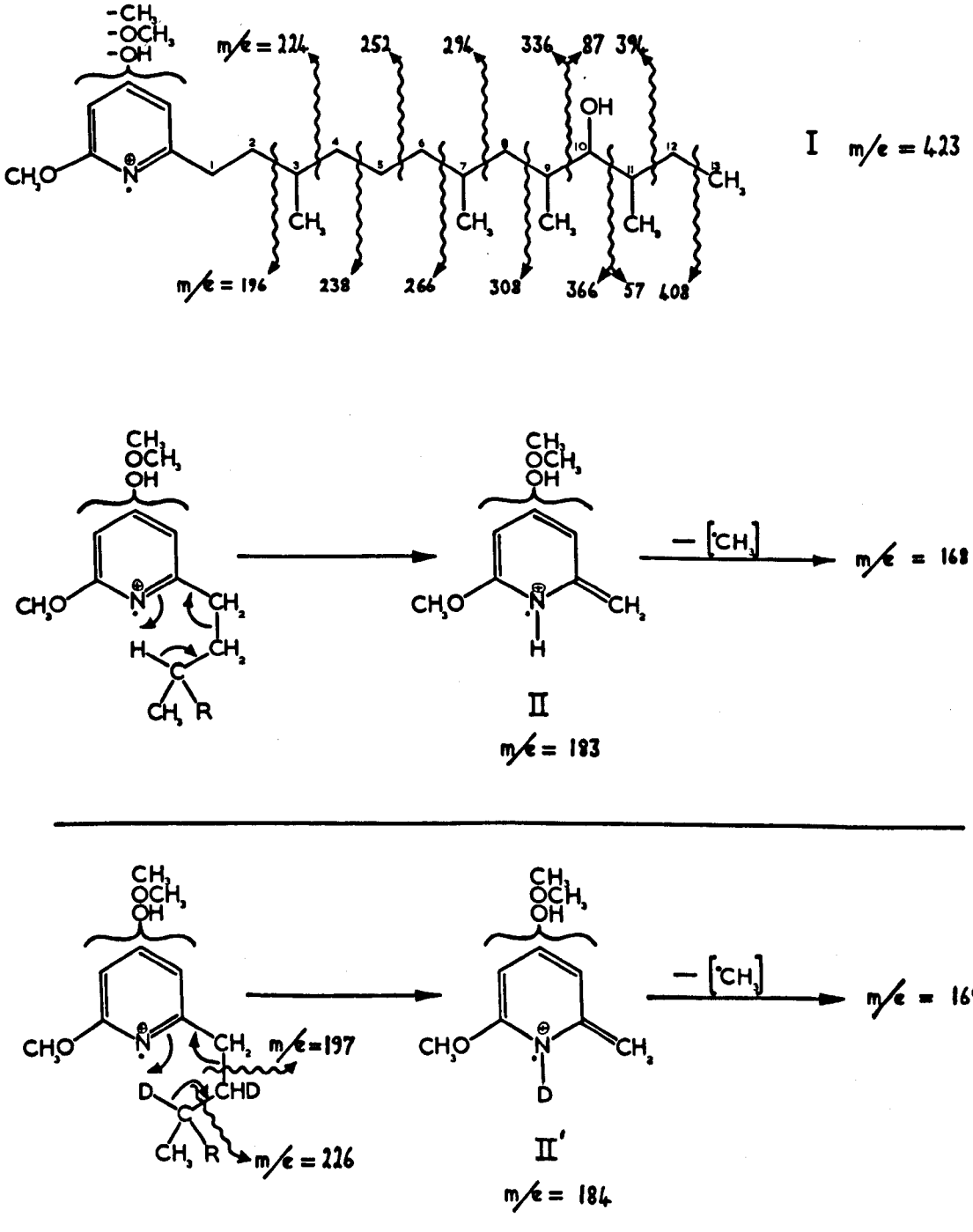
would be expected to arise from a McLafferty rearrangement, which is unlikely since there is no proton attached to C_3. Although the origin of this species remains obscure, it appears to decompose further by losing a methyl radical producing an ion at m/e = 168.

Thus the principal ionic fragments of the mass spectrum of piericidin A can all be given identity, assuming that the side chain of the molecule has the structure proposed on page 54 .

The ^{1}HNMR spectrum of piericidin A diacetate (page 137-138 and figure 35) confirms the presence of phenolic and alcoholic acetate functions in the molecule by the singlet peaks at 7.67 τ and 8.08 τ respectively. As expected, the doublet attributed to the proton

FIGURE 14

THE MASS SPECTRAL BEHAVIOUR OF OCTAHYDROPIERICIDIN A



attached to C_{10}, α to the alcoholic acetate group, is found at 5.06 τ, whereas in unacetylated piericidin A it appears at 6.36 τ. This shift is similar to that observed after the reaction of piericidin A with trichloracetylisocyanate (see page 108, 132 and figure 35). Small changes in the chemical shifts of substituents attached to the pyridine ring are also evident after acetylation as shown below.

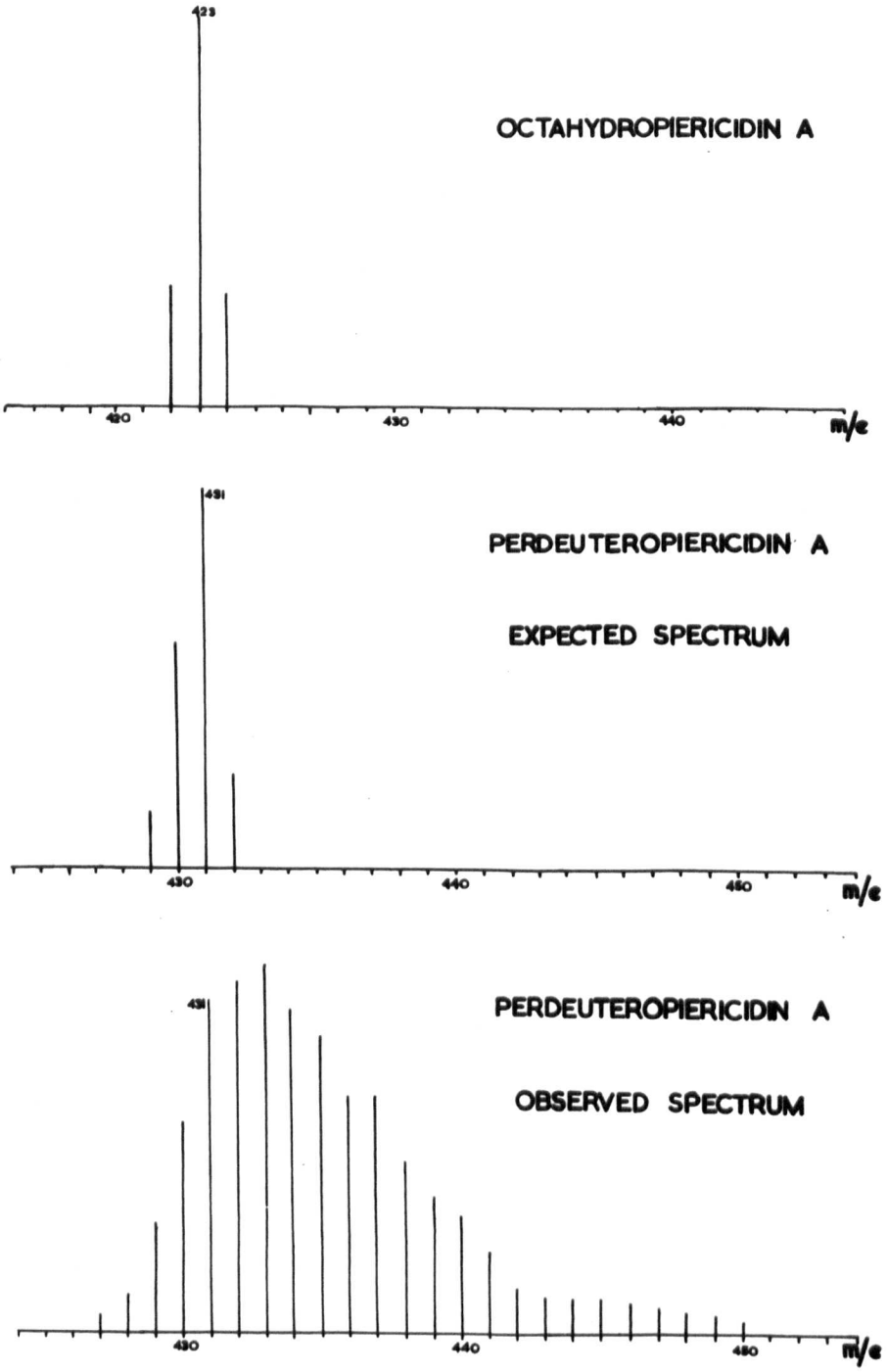
Substituent	$-OCH_3$	$-OCH_3$	$-CH_3$	$-CH_2-$
Piericidin A (τ)	6.08	6.18	7.91	6.63
Piericidin A diacetate (τ)	6.05	6.20	7.99	6.60
Shift on acetylation (τ)	−0.03	+0.02	+0.08	−0.03

Similar shifts are observed after the reaction of piericidin A and piericidin B with trichloracetylisocyanate (see pages 109, 209), but as in those cases no clear deductions can be made regarding the proximity of other substituents to the phenolic function.

The IR, UV and ^1H NMR spectra of octahydropiericidin A (page 138 and figure 36) were similar to those reported by Takahashi[2a]. In addition the mass spectrum of the compound (figure 40 of the mass spectral appendix) fully confirmed the proposed structure of its side-chain[2c,2e]. The molecular ion, I (figure 14), at m/e = 423 gives rise to ions at m/e = 408, 394, 366 and 57, 335 and 87, 308, 294, 266, 252, 238, 224 and 196 by fragmentation between consecutive carbon atoms of the side-chain, as indicated. The ion at m/e = 337 may be formed by the loss of a formylradical from that at m/e = 366, or by fission of the molecular ion between C_9 and C_{10} together with the transfer of a hydrogen atom from the alcoholic function.

FIGURE 15

THE ISOTOPIC DISTRIBUTION OF MOLECULAR IONS IN THE MASS SPECTROMETER



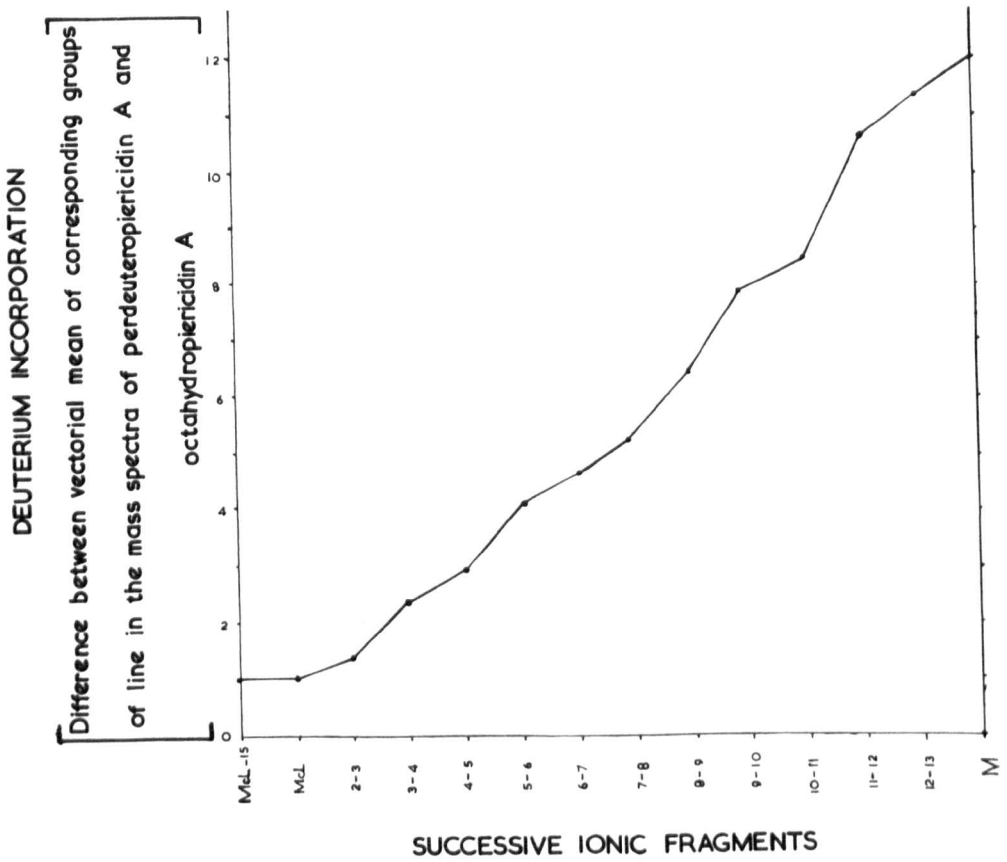
The ion at m/e = 183 can be considered to originate from a McLafferty rearrangement (I⟶II, figure 14) confirming the location of the hydrocarbon side-chain in an α position in the heterocycle (Cf page 45), giving rise in turn to that at m/e = 168 by the loss of a methyl radical. This latter process is confirmed by the existence of a peak due to the corresponding metastable transition at m/e = 154.23.

Piericidin A was reduced with deuterium in an attempt to prepare ($^2H_8$)-octahydropiericidin A, by analogy to octahydropiericidin A. The purpose of the attempt was to confirm the location of the olefinic bonds of piericidin A by a comparison of the mass spectra of its two reduction products (see page 52). Deuterium gas, prepared from deuterium oxide, was found to have an isotopic abundance of 95% (page 139). The expected and actual isotopic distributions of the molecular ion of ($^2H_8$)-octahydropiericidin A, prepared using such gas, are shown in figure 15, together with that observed in the case of octahydropiericidin A. Clearly there has been considerable incorporation of deuterium in excess of the eight atoms expected from the reduction of olefinic bonds.

The mass spectrum of the perdeutero compound (figure 40 of the mass spectral appendix) reveals a similar fragmentation pattern to that of octahydropiericidin A. Estimation of the vectorial mean of corresponding groups of spectral lines for each compound revealed that deuterium was randomly incorporated into the side-chain, apparently

FIGURE 16

THE DISTRIBUTION OF DEUTERIUM IN PERDEUTEROPIERICIDIN A



M = Molecular ion

n-(n+1) = Ion formed by fission between carbon atoms n and (n+1) of the side chain

McL = Ion formed by McLafferty rearrangement

McL-15 = Ion formed by loss of methyl radical from McL

on all carbon atoms except that adjacent to the pyridine ring. There was no incorporation of deuterium into other substituents of the pyridine ring. These results are represented graphically in figure 16.

Confirmation is provided for the position of the first olefinic bond of the side-chain by the ions at m/e = 169, 184, 197 and 226 (see figure 14, $I^1 \longrightarrow II^1$ etc.). Above m/e = 226 the isotopic distribution of deuterium obscures any such observations. One explanation of the random incorporation of excess deuterium is that double-bond migration, with concurrent incorporation of the isotope, took place on the surface of the catalyst (platinum oxide) before or during reduction. This possibility has been recognised in other systems[159].

The mass spectrum of a sample of perdeuteropiericidin A which had been dissolved in ($^2H_1$)-methanol revealed ions at m/e = 170, 185, 198 and 227 in place of those of one mass unit less (see figure 40 of the mass spectral appendix). These confirm the location of a phenolic function on the pyridine nucleus of the piericidins.

In the mass spectra of both octahydropiericidin A and perdeuteropiericidin A, peaks due to a corresponding metastable transition are detected at m/e = 154.23, 155.22 and 156.22. This corresponds in each case to the loss of a neutral fragment of mass 15 from the ion resulting from the McLafferty rearrangement at m/e = 183, 184 and 185 respectively. It has been suggested[160] that the neutral fragment is a methyl radical originating from a methoxyl group. The above evidence is consistent with this theory.

The preparation of (^{3}H)-piericidin A by the Wilzbach gas exposure technique[161], and of (^{3}H)-octahydropiericidin A from it, were straightforward (pages 143-144). The use of these compounds is discussed later (page 66-68).

The structures of metabolites C and D, whose isolation was mentioned earlier, and of the hydrogenation product of each have not been determined. However the available spectroscopic evidence of these compounds does provide some structural information (pages 134-137, 140-143). The UV spectra of the two metabolites are strikingly similar both before and after hydrogenation (see figure 37), suggesting that they are structurally closely related. The IR spectrum in solution, of each, exhibits no absorption attributable to the O-H stretching vibration of a free hydroxyl group (3,500-3,600 cm^{-1}); both have strong absorptions in the regions of 1685 cm^{-1} and 1645 cm^{-1}, possibly due to the presence of an αβ-unsaturated ester or lactone chelated to an enolic group[162]. On hydrogenation a single carbonyl absorption in the region of 1665 cm^{-1} is observed.

The mass spectra of metabolites C and D have parent ions at m/e = 236 and m/e = 206 respectively, accurate mass measurements revealing that their elemental compositions, $C_{10}H_{10}N_3O_4$ and $C_9H_8N_3O_3$ respectively, differ by CH_2O. Both molecular ions may decompose by the loss of 15 or 29 mass units, corresponding to methyl and formyl radicals. In the case of metabolite C, but not of metabolite D, the ion formed by the loss of a methyl radical from the molecular ion at m/e = 221 decomposes further by the expulsion of

carbon monoxide, producing an ion at m/e = 193. A peak due to the metastable transition corresponding to this process occurs at m/e = 168.55. The mass spectra of samples of metabolites C and D, which had been dissolved in deuterium oxide, revealed increases of one mass unit in all the above mentioned principal ions in both cases. This indicates the presence of a single exchangeable proton in both compounds, which is not lost in any of the decomposition processes mentioned above.

The molecular ions of the mass spectra of both metabolites increased by two mass units after hydrogenation, implying the reduction of a single olefinic bond in both compounds. Although it appears that both molecular ions may decompose by the loss of a formyl radical (29 mass units), the spectra are more complicated than those of the original metabolites, and preclude reliable interpretation in the absence of further corroborative evidence. The elemental compositions of the molecular ion and base peak, in the mass spectrum of reduced metabolite C, have been shown to differ by $C_2H_5O_2$ by accurate mass measurements. A sample of reduced metabolite C, which had been dissolved in deuterium oxide, revealed an increase of one mass unit in the molecular ion of its mass spectrum, indicating the presence of a single exchangeable proton as in the case of the parent compound.

An unusual feature of the mass spectra of all four compounds mentioned above concerns their molecular ions. These are of even m/e value in each case, and yet accurate mass measurements show that they contain three nitrogen atoms. The rules of valency require

an uncharged tri-aza molecule, containing carbon, hydrogen and oxygen as the only other elements, to be of odd molecular weight. One possible explanation of this phenomenon is that the four compounds are in fact salts, and that the supposed molecular ions of the spectra correspond to the cations of the salts, which are charged species before entry into the ionisation chamber of the mass spectrometer. This possibility is also consistent with their observed solubility in water, their relatively high melting points and the possibility that they may contain basic nitrogen atoms capable of forming quaternary salts.

The ^{1}H NMR spectra of metabolites C and D and of hydrogenated metabolite C are recorded (pages 135, 136, 141). In all three cases there appear from the ^{1}H NMR spectra to be more protons than are indicated by accurate mass measurements of the molecular ions in the mass spectra. This is consistent with the possibility that the compounds are salts, of which only the cations contribute to the supposed molecular ions of the mass spectra.

All three compounds exhibit a sharp singlet corresponding to one proton at -1.1τ, independent of concentration and undergoing exchange on the addition of deuterium oxide. This is probably due to the presence of an intramolecularly hydrogen-bonded phenolic or enolic proton, and may also be responsible for the observed mass spectral increases of samples dissolved in deuterium oxide. In addition metabolite C has a singlet at 8.45τ corresponding to two protons, which moves to higher field on dilution and is exchanged by the addition of deuterium oxide. This behaviour is typical of the protons of a primary amine, and, since it clearly does not feature in the supposed molecular ion of the mass spectrum of metabolite C, may be a feature of the suggested anion of this compound.

The ^{1}H NMR spectrum of metabolite C also has the following features:

(i) Two olefinic singlets each corresponding to one proton at 3.67 and 3.82 τ.

(ii) Two singlets each corresponding to three protons at 6.05 and 6.09 τ, typical of the protons of methoxyl groups attached to sp^2 hybridised carbon atoms.

(iii) One singlet, corresponding to three protons at 7.74 τ, typical of the protons of a methyl group attached to an sp^2 hybridised carbon atom.

The ^{1}H NMR spectrum of metabolite D differs slightly from these observations in that it has one more signal due to an olefinic proton and one less attributable to a methoxyl group. This coincides satisfactorily with the difference of CH_2O observed in the elemental composition of the molecular ions in the mass spectra of the two compounds (see page 59). The additional olefinic proton of metabolite D with a resonance absorption at 3.51 τ is spin-spin coupled to the olefinic proton detected at 3.67 τ, the coupling constant being 2.6 H_z. Thus it may be that the methoxyl group present in metabolite C, but absent in metabolite D, is adjacent to a single isolated olefinic proton.

On hydrogenation, two signals in the ^{1}H NMR spectrum of metabolite C, namely the olefinic singlet at 3.82 τ and the singlet at 7.74 τ, disappear. Correspondingly, new signals are found at 7.13, 8.51 and 9.1 τ, implying the reduction of an olefinic bond bearing a methyl group and a proton.

Thus the initial suspicion that metabolite C might be closely related to the piericidins, based on the observation that it produced ^{1}H NMR signals attributable to two methoxyl groups and a methyl group attached to a pyridine ring, similar to those observed in the case of the piericidins, is incorrect.

In conclusion it can be said that metabolites C and D appear to be salts. If this is the case then each has a similar ionic component, differing only in that metabolite C possesses a methoxyl group in place of an olefinic proton present in metabolite D. This common component appears to be an unsaturated nitrogen heterocycle, which, since the species was not completely hydrogenated using a platinum oxide catalyst, may possess aromatic character.

The investigation into the properties of metabolites C and D was not extended, once that it was established that they did not possess the pyridine nucleus present in the piericidins as seemed possible at one stage. The small quantities in which they were obtained also restricted their investigation.

EXPERIMENTS OF BIOCHEMCIAL SIGNIFICANCE

The potent inhibition of NADH dehydrogenase by piericidin A is s subject of considerable chemical interest (see page 25). It seems possible that the use of analogues, possessing selected chemical features of piericidin A, during inhibitory studies might lead to an understanding of the function, or lack of function, of these features during inhibition. Piericidin A has been shown to be a phenolic pyridine having a long hydrocarbon side-chain, a methyl group and two methoxyl groups as the remaining substituents[2]. A number of compounds possessing a variety of these features were tested for their inhibition of NADH linked oxidation in mitochondria (pages 146-147). Some of these were synthesised (pages 180-198).

Piericidin B and octahydropiericidin A were found to be almost as potent as inhibitors as piericidin A, indicating that the side-chain need not possess an alcoholic function nor be unsaturated in order to achieve complete inhibition.

Thus the scheme shown in figure 8 (see also pages 26-27) involving an olefinic linkage of the side-chain, does not account for the inhibition by these piericidins, and consequently the proposed chemical tests of this scheme involving model systems are unnecessary.

Piericidin A diacetate was found to be three orders of magnitude less inhibitory than piericidin A, suggesting that the phenolic function of piericidin A is of considerable importance during inhibition.

Of the remaining compounds all were more than 10^5 times less effective as inhibitors than piericidin A. However, of these,

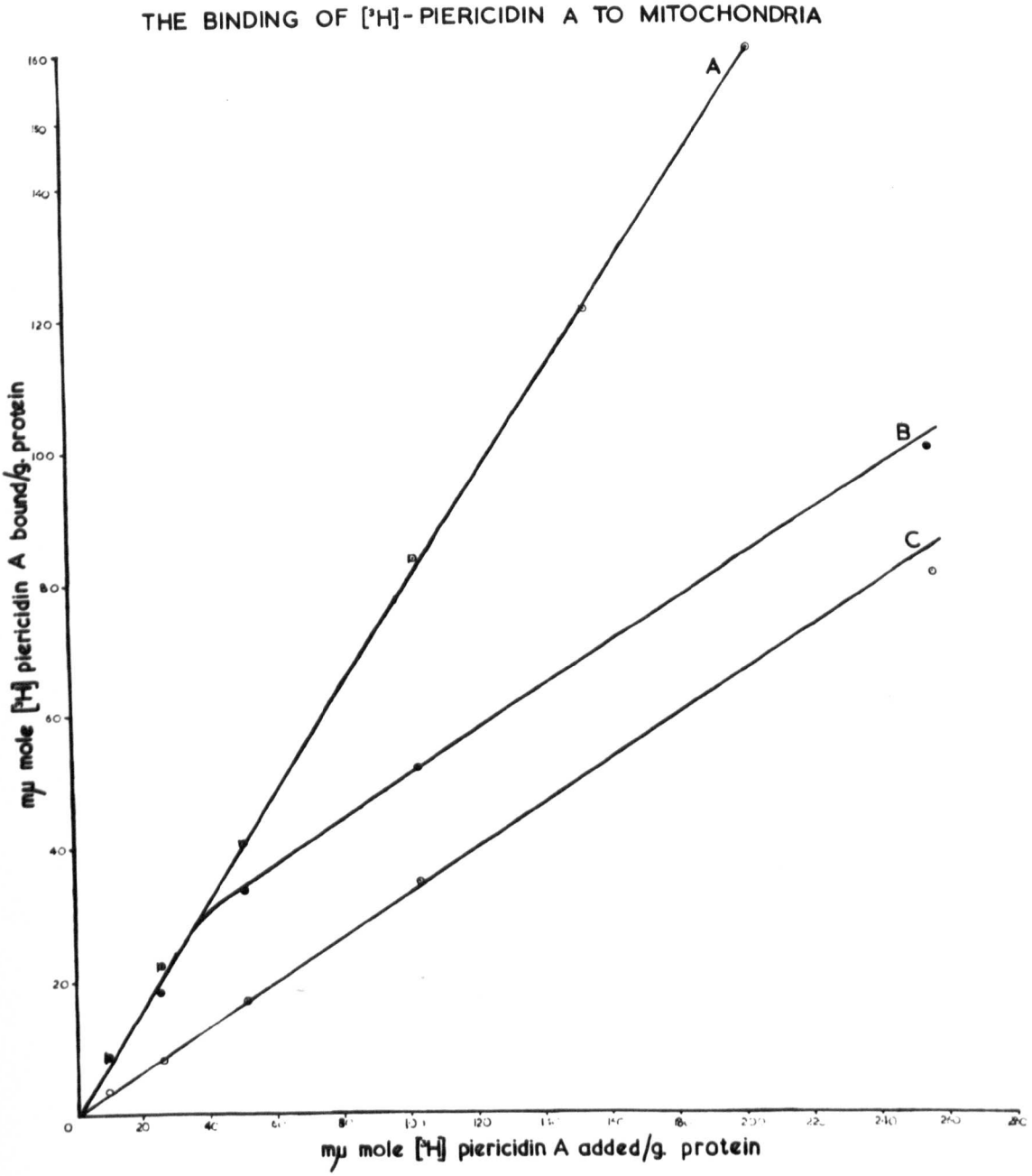
compounds having a lipophilic side-chain and a phenolic function were the most effective. This provides some confirmation for the functional importance of the phenolic moiety suggested above in the case of the piericidins. The importance of lipid solubility has already been recognised (page 25).

The significance of these two features is exemplified by the three compounds 2-(1-n-hexyl)-4-hydroxypyridine, 2-(1-n-hexyl)pyridine and 4-hydroxy-2-methylpyridine. The first is required in a concentration 3.5 times less than the second and 50 times less than the third in order to achieve the same degree of inhibition. Furthermore 2-(1-n-hexyl)-4-methoxypyridine and 4-chloro-2-(1-n-hexyl) pyridine are both less inhibitory than 2-(1-n-hexyl(-4-hydroxypyridine in accordance with the suggested importance of the phenolic function during inhibition. Considering the similarity of this latter compound to the published structure of piericidin A[2,3], its comparative impotency as an inhibitor is a matter for some surprise.

The importance of lipid solubility concerning these inhibitors is again illustrated by the examples of 4-(1-n-hexyl)-2-methoxyphenol and vanillin, the former being 66 times more potent than the latter. 4-(1-n-hexyl)-2-methoxyphenol is also more potent than 4-(1-n-pentyl) phenol, a fact which may be due to the presence of either the methoxyl group or the longer side-chain in the former.

It is concluded from these studies using analogues that the phenolic function and the long side-chain of the piericidins are both essential in order to achieve potent inhibition of NADH

FIGURE 17



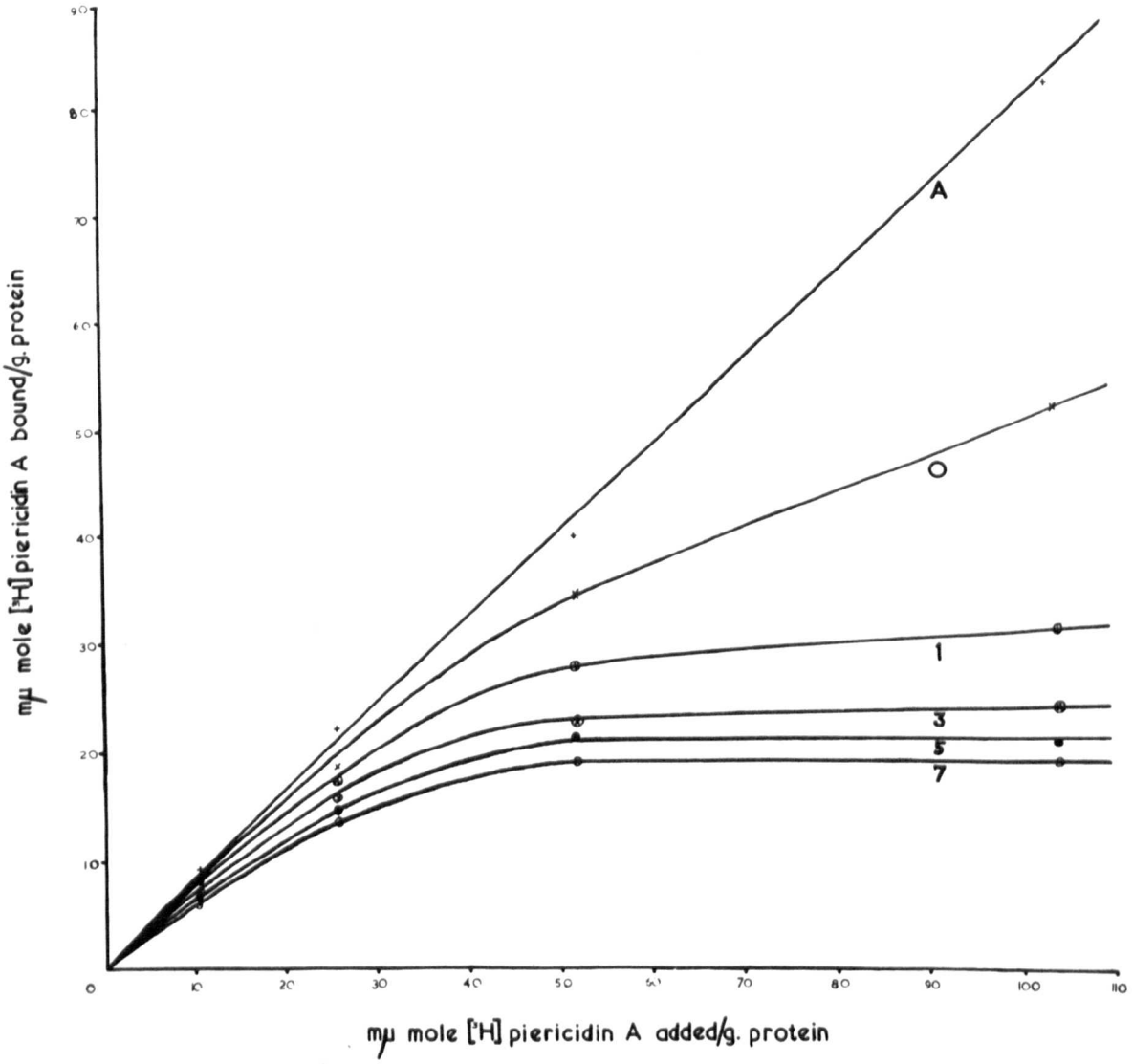
Curve A ; binding in sucrose-tris medium.

Curve B ; binding in sucrose-tris-BSA medium.

Curve C ; binding in sucrose-tris-BSA medium after preincubation with unlabelled piericidin A.

FIGURE 18

THE EFFECT OF REPEATED WASHING ON THE BINDING OF [^{3}H]-PIERICIDIN A TO MITOCHONDRIA



Curve A; binding in sucrose-tris medium.

Curves O, 1, 3, 5, 7; binding, after washing in sucrose-tris-BSA medium the indicated no. of times.

dehydrogenase.

A sample of piericidin A labelled with tritium (page 143) was used to investigate the binding of piericidin A to mitochondria. The binding of (^{3}H)-piericidin A to mitochondria extended to concentrations above those required for the maximal inhibition of NADH oxidation (page 148 and curve A figure 17). Hence, in agreement with earlier observations (see page 20), piericidin A is not a specific inhibitor of NADH dehydrogenase. In the presence of 2% bovine serum albumen the binding curve became clearly biphasic (curve B figure 17). Furthermore the rapidly rising portion of this curve could be abolished by titrating the mitochondria to maximal inhibition with unlabelled piericidin A before the addition of any (^{3}H)-piericidin A (curve C figure 17). A measure of the concentration of piericidin A involved at the sensitive site was obtained by subtracting curve C from curve B and was in the region 0.02 mμ moles/mg. of protein. Repeated washing of the mitochondria with 2% bovine serum albumen also indicated that a similar amount of (^{3}H)-piericidin A was tightly bound (figure 18). A comparison of the amount of (^{3}H)-piericidin A bound after repeated washing and the degree of inhibition obtained showed a direct correlation (see page 149).

It was shown that piericidin A was not removed from the specific binding site by equilibration with unspecifically bound piericidin A, since the addition of excess

unlabelled piericidin A to mitochondria previously treated with (^{3}H)-piericidin A resulted in no displacement of (^{3}H) piericidin A after repeated washing (page 151). Conversely when excess (^{3}H)-piericidin A was added to mitochondria pretreated with unlabelled piericidin A, the (^{3}H)-piericidin A was removed by repeated washing (page 150).

Pretreatment of mitochondria with rotenone, amytal or unlabelled piericidin A decreased the binding of (^{3}H)-piericidin A (page 151), indicating that all three compounds compete for a common binding site. This is consistent with the knowledge that they all inhibit respiration in the same region of the respiratory chain (see pages 21-22). Antimycin A on the other hand, an inhibitor acting elsewhere in the respiratory chain (see figure 2), did not decrease the binding of (^{3}H)-piericidin A.

The extraction of mitochondria, treated with (^{3}H)-piericidin A, with acetone resulted in complete removal of the tightly bound radioactivity. The extract contained the expected mitochondrial lipids[163] together with unchanged (^{3}H)-piericidin A identified chromatographically (page 153). Hence although piericidin A may interact with mitochondrial lipids, and with other mitochondrial components, it does not become covalently bound to them.

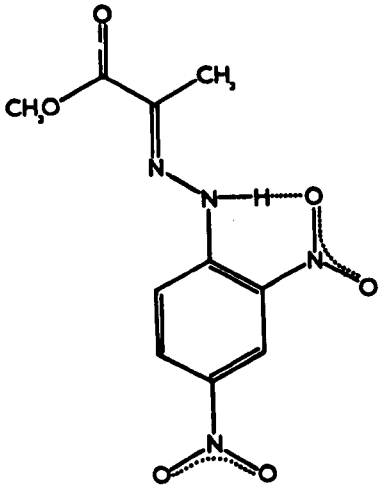
The possibility that piericidin A might interact with the functional groups of proteins was investigated in a rudimentary manner by heating (^{3}H)-piericidin A with appropriate amino acids in solution (page 154), and examining the products by thin layer

chromatography. In all cases about 80% of the original radioactivity was recovered at the R_f corresponding to piericidin A and at the origin, negligible amounts being found elsewhere. The radioactivity detected at the origin was assumed to be due to decomposed piericidin A, since it was found in a blank experiment. Only in the case of cysteine hydrochloride, and to a lesser extent of cysteine itself, was the ratio of radioactivity at the origin to that corresponding to unchanged piericidin A significantly different from the blank experiment. Thus despite the fact that piericidin A might be expected to behave either as a weak acid or a weak base, no interaction between it and free amino or carboxyl functions was detected. The results with cysteine and its hydrochloride probably indicate that oxidation of the sulphydryl groups in these compounds has involved the olefinic linkages of piericidin A in a destructive manner, rather than any reaction of biochemical significance.

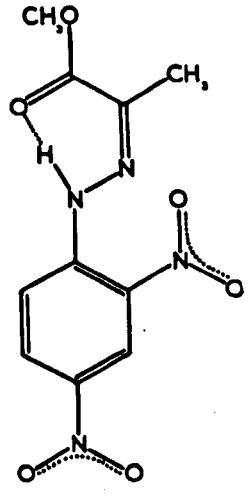
Following the observation that 2,5-diethoxy-1,4-benzoquinone can act as a charge transfer donor[164] towards 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)[165], and towards tetracyanoethylene (TCNE)[166], analogous experiments were performed with ubiquinone and piericidin A (page 155), in the hope of finding a property common to the two compounds of some biochemical significance. Charge transfer was not detected in either case, nor surprisingly was it detected between tetramethoxy-1,4-benzoquinone and DDQ or TCNE. It is concluded that piericidin A and ubiquinone are not likely to act as charge transfer donors in biochemical systems.

FIGURE 19

2,4-DNP DERIVATIVES OF METHYL PYRUVATE

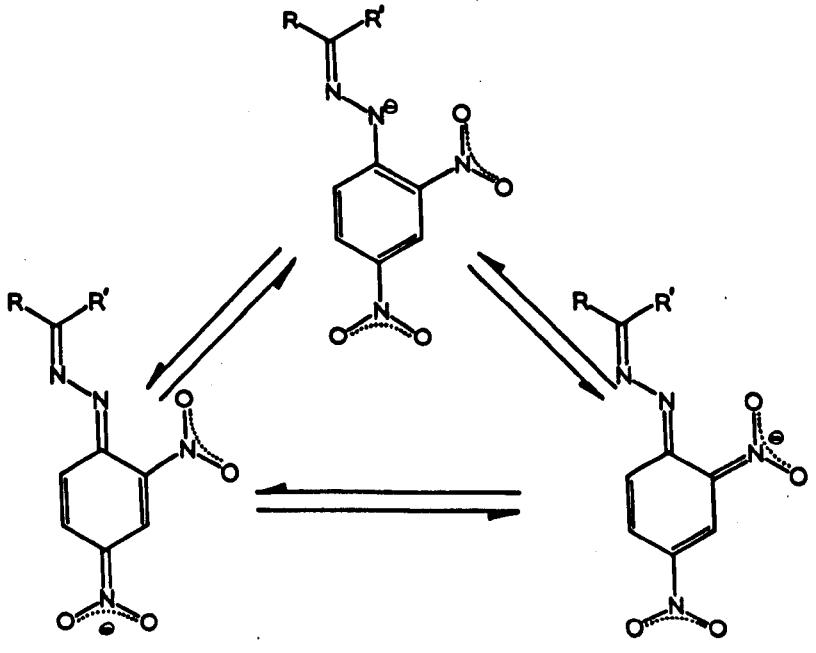


I



II

THE CHROMOPHORIC ANION OF 2,4-DNP DERIVATIVES



THE 2,4-DINITROPHENYLHYDRAZONES OF METHYL PYRUVATE

The discrepancy in the reported melting point [148,149,150] of the 2,4-dinitrophenylhydrazone of methyl pyruvate was resolved by the isolation and characterisation of two distinct isomers of this compound.

Corresponding isomers of 2,4-DNP derivatives of other pyruvic acid esters have previously been assigned the structures I and II (figure 19), and referred to as α and β forms[150]. These assignments were based on comparisons of the UV spectra with compounds in which the proposed isomerisation is not possible.

Isomerism has also been observed in the 2,4-DNP derivatives of α-keto acids[151]. In these cases the IR spectra indicated hydrogen bonding to the carbonyl function in one isomer, but not in the other. Furthermore the addition of sodium hydroxide solution to the "hydrogen-bonded" isomer had very little effect, whereas in the other case a red colour was immediately produced.

Methyl pyruvate was produced from pyruvic acid by the action of diazomethane[167] (page 157). A 2,4-DNP derivative of methyl pyruvate was then prepared by reaction with 2,4-dinitrophenylhydrazine according to a standard method[168] (page 157). It was identified as the α form by comparison of its melting point and ultra violet absorption spectrum with reported values[150]. In dilute, slightly acidic, methanolic solution this isomer became partially converted to a second isomer on prolonged exposure to sunlight (page 158). The second, or β, isomer was separated chromatographically.

Some of the chief differences in the properties of the two isomers are shown in Table 1, however considerable similarities, notably in the mass spectra and parts of the NMR and IR spectra, also exist between them.

Table 1 Some properties of the two 2,4-DNP derivatives of methyl pyruvate

	α Isomer	β Isomer
Appearance	yellow crystalline	orange crystalline
M.Pt. °C	186–187	164.5–165.5
ν N-H (nujol mull) cm^{-1}	3150 (med. st.)	3310 (med. st.)
ν C=O (nujol mull) cm^{-1}	1700 (st.)	1730 (st.)
λ max (methanol) nm.	361 (17,400)	347 (22,800)
τ NH ($CDCl_3$, 1%)	-4.1	-1.0
T.L.C. (System)	R_f = 0.43	R_f = 0.16
Effect of NaOH on methanolic solution	No colour change	Red colour produced

It appears that hydrogen bonding between the proton attached to nitrogen and the ester carbonyl function is present in the α, but not the β isomer. This specifically accounts for:

1) the C=O stretching vibration frequency being 1700 cm^{-1} instead of 1730 cm^{-1}. (In methyl pyruvate a value of 1735 cm^{-1} is observed).

2) the N-H stretching vibration frequency being 3150 cm^{-1} instead of 3310 cm^{-1}.

3) the nuclear magnetic resonance of the proton attached to nitrogen being at -4.1 τ instead of - 1.0 τ.

The difference in behaviour towards base is less readily explained. The red colour is reversibly produced, and is also

observed with the 2,4-DNP derivative of acetone, suggesting that this latter compound structurally resembles the β rather than the α isomer. The chromophone may simply be produced by loss of the proton attached to nitrogen. Such an anion could have azaquinonoid character responsible for the chromophone, (see figure 19). In the case of the "hydrogen bonded" α isomer the loss of the proton might be expected to be less facile, accounting for the difference. m-Dinitrobenzene produces no colour under these conditions, showing that the effect is not solely connected with the aromatic ring.

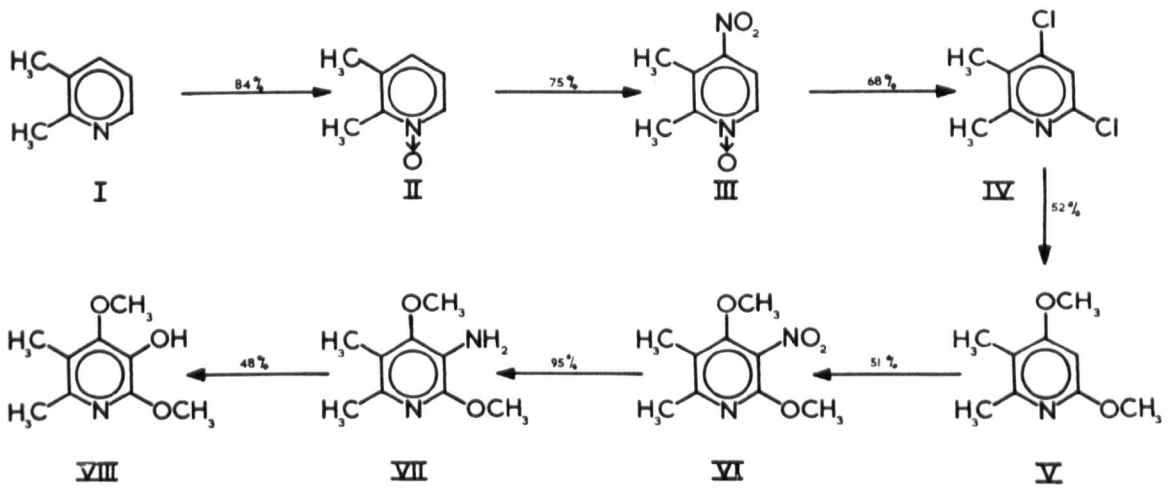
It is concluded that the α isomer of the 2,4-DNP derivative of methyl pyruvate has a structure represented by II (figure 19), and the β isomer a structure represented by I (figure 19). This conclusion is contrary to the previous report[150], and can be extended to include the whole series of pyruvic acid ester derivatives mentioned in this report.

It can be seen from the above results that neither of the isolated 2,4-dinitrophenylhydrazones of methyl pyruvate have a melting point corresponding to that of the compound reported by Takahashi[2b], obtained on treatment of an ozonolysis product of "O-methylhydropiericidin A" with Brady's reagent. Thus this compound was not a 2,4-dinitrophenylhydrazone of methyl pyruvate as was claimed. The conclusions made from this claim, concerning the relative positions of substituents in the pyridine ring of piericidin A, must therefore be discounted. It is proposed that the correct

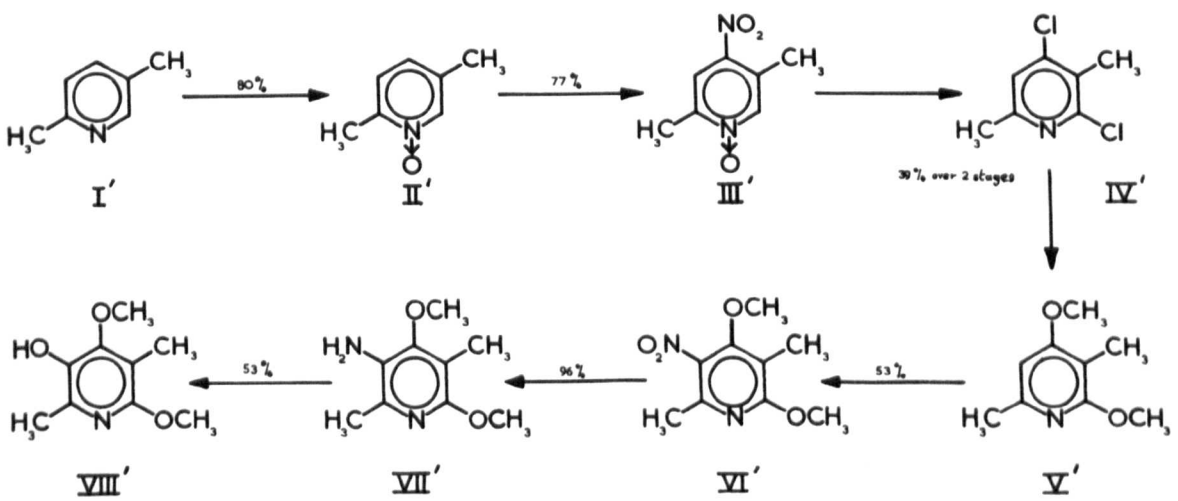
structure of this pyridine ring may be any one of the six isomers represented in figure 12, but that it is more likely as mentioned earlier to be a 3-hydroxypyridine than a 4-hydroxypyridine.

FIGURE 20

SYNTHETIC ROUTES



overall yield I→VIII = 5·2% (7 stages)



overall yield I′→VIII′ = 6·2% (7 stages)

THE SYNTHESIS OF 4,6-DIMETHOXY-2,3-DIMETHYL-5-HYDROXYPYRIDINE AND OF 4,6-DIMETHOXY-2,5-DIMETHYL-3-HYDROXYPYRIDINE

The synthesis of the two above named β -hydroxypyridines, both possible candidates for the correct structure of the pyridine nucleus of piericidin A (see page 51 and figure 12, V and VI where R = H), has been achieved. The synthetic routes, which are from the corresponding lutidines and are analogous, have the advantage of being based on known reactions which do not accommodate ambiguous products, but they are not the only possible synthetic pathways and are not necessarily the shortest. They are summarised in figure 20.

The first two stages in each case were by standard procedures[169] (see pages 160, 173-174), since both dimethyl-4-nitropyridine-1-oxides (III and III[1] figure 20) are reported in the literature[170,171].

2,4-Dichloropyridine has been prepared from 4-nitropyridine-1-oxide by the action of sulphuryl chloride in a sealed tube[172]. This method was repeated using phosphorus oxychloride under reflux with an almost identical yield of the dichloropyridine (page 161). It was therefore decided to adopt the latter technically safer procedure in analogous reactions involving the two dimethyl-4-nitropyridine-1-oxides (III and III[1], figure 20).

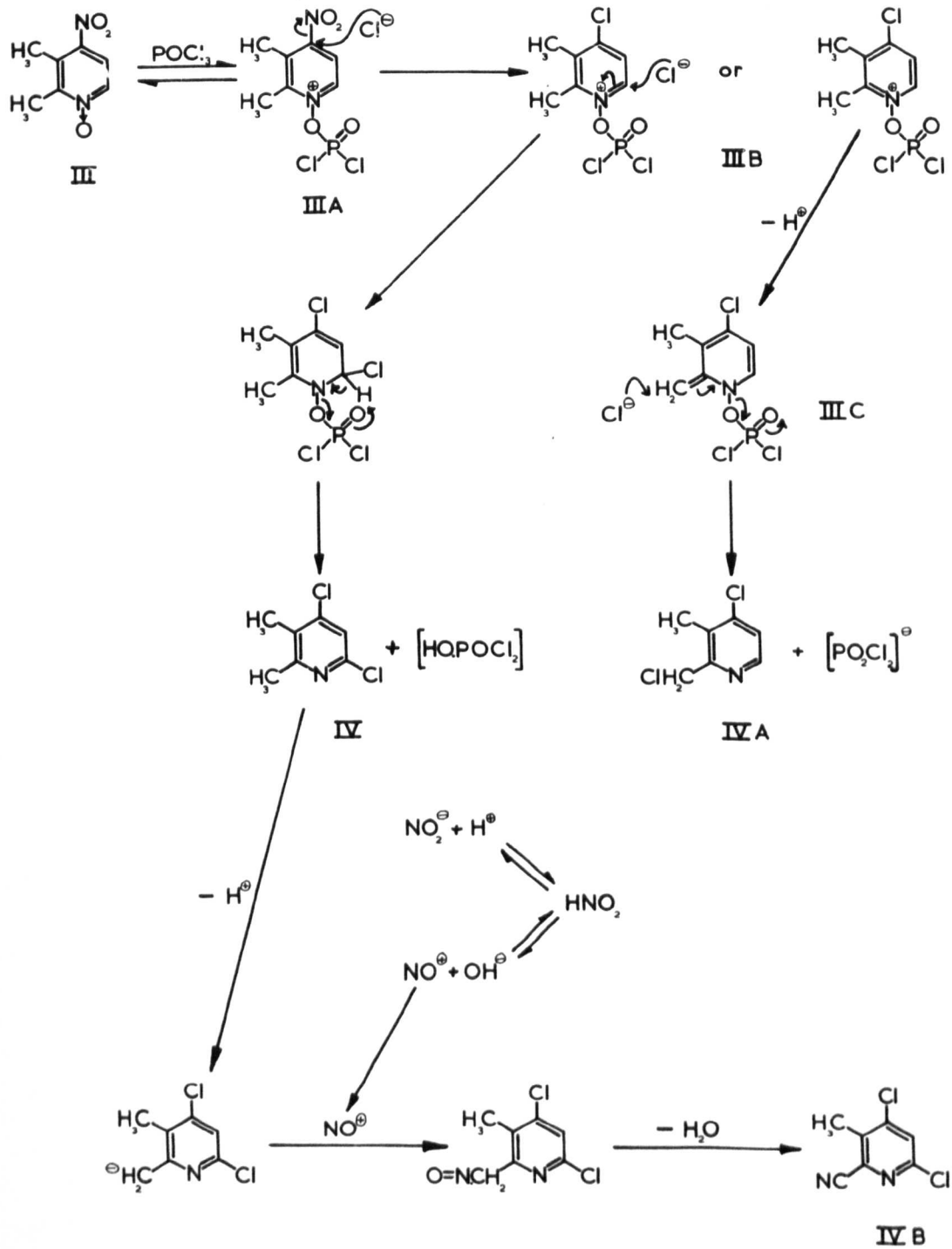
The reaction product of 2,3-dimethyl-4-nitropyridine-1-oxide was examined in detail (pages 161-164), three compounds being isolated. Each was identified as a dichloropyridine from the isotopic distribution of the molecular ion in its mass spectrum. The two major components, 4,6-dichloro-2,3-dimethylpyridine and 4-chloro-2-

chloromethyl-3-methylpyridine isolated in the ratio 2·6:1, were isomeric and were distinguished principally by their ^{1}H NMR spectra. These are interpreted in figure 23 (I and II). An interpretation of their mass spectra is presented in figure 46 of the mass spectral appendix. The molecular ion of 4,6-dichloro-2,3-dimethyl-pyridine is also the base peak of the spectrum, but in the case of 4-chloro-2-chloromethyl-3-methylpyridine the base peak corresponds to an ion formed by the facile elimination of a chlorine atom from the molecular ion. Presumably this is the chlorine atom of the 2-chloromethyl group. The stability of the resultant ion may be explained as indicated in figure 46.

The third component, 2-cyano-4,6-dichloro-3-methylpyridine possessed an IR absorption at 2240 cm^{-1}, typical of the C≡N stretching vibration of an aromatic nitrile. Since the ^{1}H NMR spectrum indicated the presence of only one aromatic proton and one methyl group in the molecule (interpretation in figure 23, III), the other carbon substituent, formerly a methyl group, had clearly been converted to a cyano group, as detected independently. It was concluded that this cyano group was in the α rather than the β position for two reasons. Firstly, by comparison with the assignments given to compounds I and II in figure 23, the protons of an α-methyl group in a dichlorocyanopyridine might be expected to have a resonance frequency below that of 7.46 τ observed. Secondly on chemical grounds (see below) an α-cyano group is more likely. The mass spectrum of this compound is interpreted in figure 46 of the mass spectral appendix.

FIGURE 21

MECHANISMS OF REACTION



The initial step in the production of the above mentioned products is probably the formation of a salt with phosphorus oxychloride[173] (figure 21, IIIA). Nucleophilic displacement of the nitro group by chloride ion will release nitrite ion producing IIIB. Nucleophilic attack by chloride ion on this species at the unsubstituted α position, followed by elimination of the elements of phosphorodichloridic acid may be considered to produce the desired product IV. On the other hand, loss of a proton from III B to produce the methide, III C, may then give 4-chloro-2-chloromethyl-3-methyl-pyridine, IVA, by nucleophilic attack of chloride ion on the methide.

The chlorination of ɣ or α methyl groups in pyridine-1-oxides using phosphorus oxychloride has been observed before[174], however the formation of a cyanopyridine is more unusual. The additional nitrogen atom can only be derived from the nitro group present in the starting material III (figures 20 and 21). It is proposed that nitrite ions produced during the reaction are in equilibrium with nitrosonium ions (NO^+), the equilibrium being in favour of the latter since the medium is acidic. Nitrosation of the most acidic methyl group may then occur, followed by the loss of water in the hygroscopic medium, producing the product IV B. It is assumed that the α methyl group is more acidic than the β methyl group, in line with general observations on methyl pyridines[175,176]. It is conceivable that the methide, III C, might act in a nucleophilic manner towards nitrosonium ions, producing the same product, IV B, but this is considered less likely since it requires the methide to act in an ambident manner under a single set of experimental conditions.

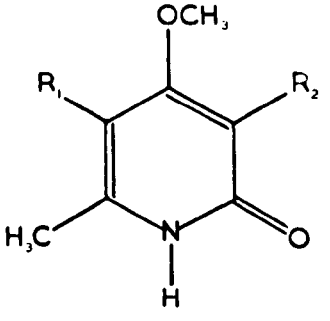
Clearly the proposed nitrosation cannot occur before the replacement of the nitro group in III A, a process which concurrently reduces the acidity of the β-methyl group. The possibility of an intramolecular reaction involving the β-methyl and 4-nitro groups is considered unlikely, since nitrous acid is quite clearly present in solution, as apparent from the observation of oxides of nitrogen into which it decomposes. An intermolecular mechanism is also favoured by the fact that on using a greater proportion of phosphorus oxychloride for the reaction, negligible amounts of the cyano compound were obtained (see note on page 162).

The reaction of 2,5-dimethyl-4-nitropyridine-1-oxide with phosphorus oxychloride was not examined in detail, the crude reaction product being continued to the next stage (page 174). However the products of this next reaction stage indicated that 4,6-dichloro-2,5-dimethylpyridine and 4-chloro-2-chloromethyl-5-methylpyridine had been produced in a ratio of 4:1. No cyano compound was detected in this case, probably on account of the greater proportion of phosphorus oxychloride used during the reaction.

Mixtures of 4,6-dichloro-2,3-dimethylpyridine with 4-chloro-2-chloromethyl-3-methylpyridine and of 4,6-dichloro-2,5-dimethylpyridine with 4-chloro-2-chloromethyl-5-methylpyridine were treated with sodium methoxide in methanol, in a manner similar to procedures recorded in the literature[172a,177] (pages 164,174). The expected products, corresponding to nucleophilic displacement of chlorine by methoxide ion (including V and V^1, figure 20), were isolated in each case.

FIGURE 22

POSSIBLE STRUCTURE OF A PRODUCT OF REACTION



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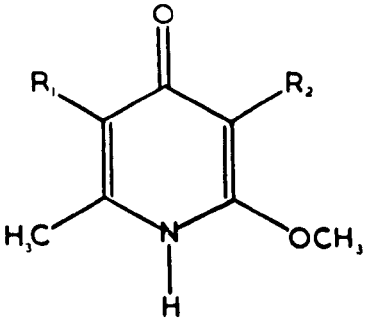
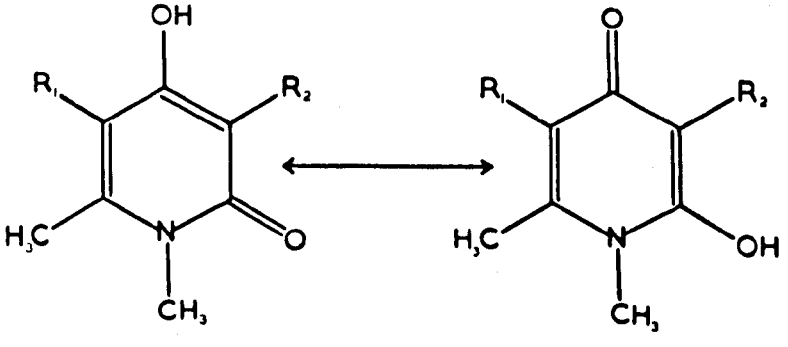


II



III

$R_1 = CH_3$, $R_2 = H$ or $R_1 = H$, $R_2 = CH_3$

Their ^{1}HNMR spectra were completely consistent with the proposed structures and are interpreted in figure 23 (IV to VII). Their mass spectra (see mass spectral appendix, figure 44) are interpreted in terms of their proposed structures (see mass spectral appendix figure 47). The ease with which a hydrogen radical is lost from the molecular ions of 4,6-dimethoxy-2,3-dimethylpyridine and 4,6-dimethoxy-2,5-dimethylpyridine appears to be typical of α-methoxy-pyridines (page 117). The facile loss of 30 mass units from the molecular ions of 4-methoxy-2-methoxymethyl-3-methylpyridine and 4-methoxy-2-methoxymethyl-5-methylpyridine is most probably due to the elimination of formaldehyde via a McLafferty rearrangement (see mass spectral appendix figure 47).

In addition to the two expected compounds mentioned above a further product was isolated in each case, the two products being of equivalent structure, judging from their spectral similarities (pages 166 , 176). Each possessed a strong IR absorption in the 1630-1660 cm^{-1} region indicative of a pyridone (see page 37). In addition the ^{1}HNMR spectra showed peaks attributable to one exchangeable proton, two methyl groups and a proton each attached to an sp^2 hybridised carbon atom, and either a methoxyl group attached to an sp^2 hybridised carbon atom or an N-methyl group of 1-methyl-pyridone. This information can be accommodated by three different structures (see figure 22, I, II and III), each consistent with the elemental analyses and the mass spectra. Since the UV spectra remain unchanged in neutral and basic solution and exhibit a hyprochromic shift in acid solution, neither compound is likely to be

a 1-(H)-2-pyridone or a 1-(H)-4-pyridone (see pages 34 , 36). A compound with structure III (figure 22) might conceivably have UV absorptions of the type observed, although in the absence of any analogy no such assumption can be made. That this third structure was correct was deduced from two further experiments with the 2,3-dimethylpyridine derivative. Firstly reaction with trichloroacetylisocyanate revealed two products by ^{1}H NMR spectroscopy (page 167), the homogeneity of the starting material being well established. It appears from other experiments (page 110) that trichloroacetylisocyanate reacts with tautomeric hydroxypyridines predominantly at the oxygen atom, producing a single product. It is therefore concluded that in this case, since there are two products, reaction has taken place at two oxygen atoms rather than at an oxygen and a nitrogen atom. This favours structure III (figure 22). Secondly, if the compound contained a methoxyl group, then it should be possible to hydrolyse this function producing a dihydroxypyridine. Three different attempts at ether cleavage based on established procedures[106a, 178 179] failed to produce any such product, substantial recoveries of unchanged starting material being achieved in each case (pages 167-168).

It is therefore proposed that the two compounds are 4-hydroxy-1,2,3-trimethyl-6-pyridone and 4-hydroxy-1,3,6-trimethyl-2-pyridone, existing as such in their principal tautomeric forms[106] (page 31). The hydrolysis implied in their formation probably occurs during the experimental work-up, the rearrangement to 1-methylpyridone structures being more likely to occur thermally during the reaction.

The desired products V and V[1] (figure 20) of the above reactions were easily separated in quantity chromatographically.

Both of these compounds were readily nitrated using mixed acids[180] producing over 50% of the desired products VI and VI[1] (pages 168, 177). The identity of the products was consistent with their ^{1}NMR spectra, which are interpreted in figure 23 (VIII and IX), and with their elemental compositions and the molecular ions of their mass spectra. No starting material was recovered in either case, and only in the case of the reaction involving 4,6-dimethoxy-2,3-dimethylpyridine was any other product isolated, even that being only in a 0.5% yield (page 169). This compound was tentatively identified as 2-cyano-4,6-dimethoxy-3-methylpyridine from its IR absorption at 2240 cm^{-1}, typical of the C-N stretching vibration of an aromatic nitrile, and from its ^{1}H NMR spectrum, which is interpreted in figure 23 (X).

The ease of the above nitration reactions (30 mins. at 0-20°C), compared to that of pyridine[181], is a measure of the change in character of the pyridine nucleus brought about by four electron donating substituents. The remaining unrecovered material in both of the nitration reactions was water soluble. This may indicate that some cleavage of the methoxyl groups of the aromatic nucleus, only to be expected in acid solution, has occurred.

Catalytic hydrogenation of the two nitro-compounds prepared above resulted in the isolation of the corresponding amino-compounds in essentially quantitative yields. 5-amino-4,6-dimethoxy-2,3-dimethylpyridine and 3-amino-4,6-dimethoxy-2,5-dimethylpyridine (VII and VII[1] in figure 20) were clearly identified from their

spectral properties (pages 171, 178). Both possessed IR absorptions in the region of 3350 cm^{-1} and of 3450 cm^{-1} attributable to the N-H stretching vibrations of a primary amino group. The exchangeable singlet between 5.7 and 5.9 τ corresponding to two protons, found in the ^1H NMR spectrum of each compound in solution in d_6-DMSO can similarly be attributed to the protons of the amino group. Interpretations of the ^1H NMR spectra of each compound in deuterochloroform are shown in figure 23 (XI and XII). The molecular ions of each compound in the mass spectrometer decomposed principally by the elimination of 1, 15, 43 or 30 mass units, corresponding to hydrogen, methyl or acetyl radicals, or formaldehyde respectively. Possible structures of the resultant ionic species are shown in figure 48 of the mass spectral appendix.

The final stage in the synthesis of the two β-hydroxypyridines related to the piericidins (VIII and VIII[1], figure 20) involved the hydrolysis of the diazonium salts derived from the above amines. The initial attempt to do this, based on the procedure of an analogous reaction, itself of low yield[182], produced none of the desired product (page 173). Instead a bright red oil was isolated, having a strong IR absorption at 2160 cm^{-1}, typical of the N≡N stretching vibration of a diazonium salt. Since no hydrolysis or other change occurred in boiling 1M sulphuric acid, the compound is more likely to have been an azo dye.

The experiment was repeated at a lower temperature using 1M sulphuric acid, with the reactants in much more dilute solution and allowing a longer time for reaction. The reaction was followed spectrophotometrically, and using a spot test for diazonium compounds (see figure 39). It appeared to be completed after 20 hours. In both cases, yields of the desired hydroxypyridine (VIII and VIII^1, figure 20) were near 50% (pages 171, 179). The improvement may have been due both to the stronger acidity of the solution, suggesting the involvement of a protonated species in the reaction[183], and to the reduction of by-products as a result of dilution.

In chloroform solution both hydroxypyridines absorbed IR radiation strongly at 3550 cm^{-1}, a wavenumber typical of the O-H stretching frequency of free phenolic groups in pyridinols (see pages 37-38). Both displayed variations of their UV spectra in acidic, basic and neutral solutions analogous to those of 3-hydroxypyridine itself (see pages 35, 210). Their ^{1}H NMR spectra (interpreted in figure 23, XIII and XIV), mass spectra (see figures 45 and 49 of the mass spectral appendix, and page 120 for an interpretation) and elemental analyses were entirely consistent with their proposed structures.

The yields of each stage in the two synthetic sequences are shown in figure 20, the overall yield after seven stages being very similar in each case at just over 5% and just over 6%.

It is interesting to note that, using the available spectral information, it would have been practically impossible to distinguish the isomeric compounds IV and IV^1, V and V^1, VI and VI^1, VII and VII^1

and VIII and VIII[1] (figure 20) without knowing from which lutidine they had been synthesised. Minor differences were observed, for instance, in the UV, ^{1}HNMR and mass spectra of the two hydroxypyridines VIII and VIII[1] (figure 20). However these do not lend themselves to interpretation in a manner leading to predictions concerning the spectra of similar isomers. In particular it would be difficult to forecast the spectral differences by which compounds having the four remaining possible structures of the pyridine nucleus of the piericidins (I-IV, figure 12) could be distinguished, assuming that they all existed predominantly as pyridinols. However, it seems likely that two of these, I and II, would exist predominantly as 1-(H)-4-pyridones (page 51), whereas III and IV would be expected to exist as pyridinols. 1-(H)-4-pyridones and pyridinols are easily distinguished spectrally (pages 42-43).

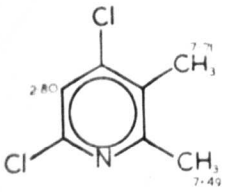
Since piericidin A behaves as a pyridinol (page 46), and does not correspond to either of the synthesised isomeric hydroxypyridines, V and VI (figure 12,R=H), it may be deduced that either structure III or structure IV (figure 12) corresponds to that of the pyridine nucleus of piericidin A.

Comparisons between the two synthesised hydroxypyridines V and VI (figure 12,R=H), piericidin A and other hydroxypyridines are considered more fully on pages 95-124.

FIGURE 23

^{1}H NMR ASSIGNMENTS

τ ($CDCl_3$)



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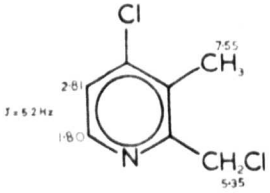

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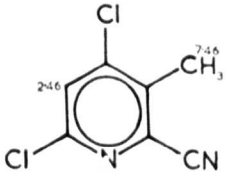
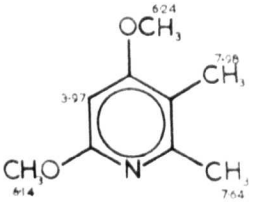
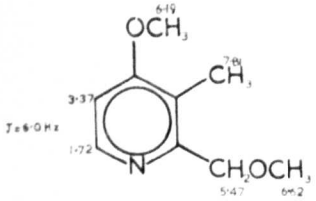


III



IV



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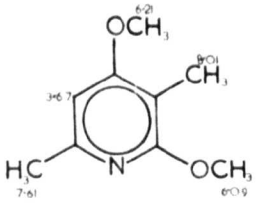

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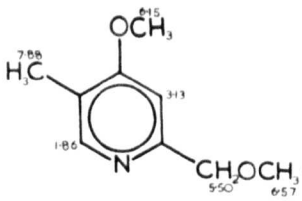
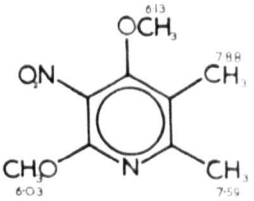
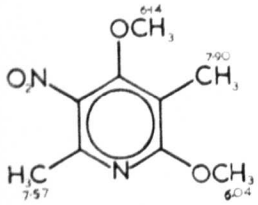


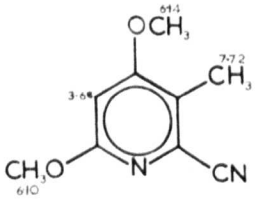
VII



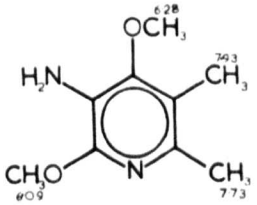
VIII



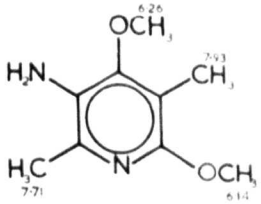
IX



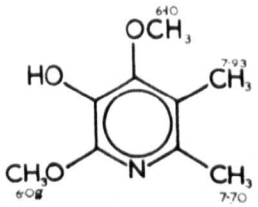
X



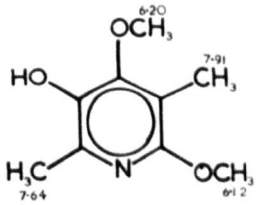
XI



XII



XIII



XIV

FIGURE 24

SYNTHETIC ROUTES

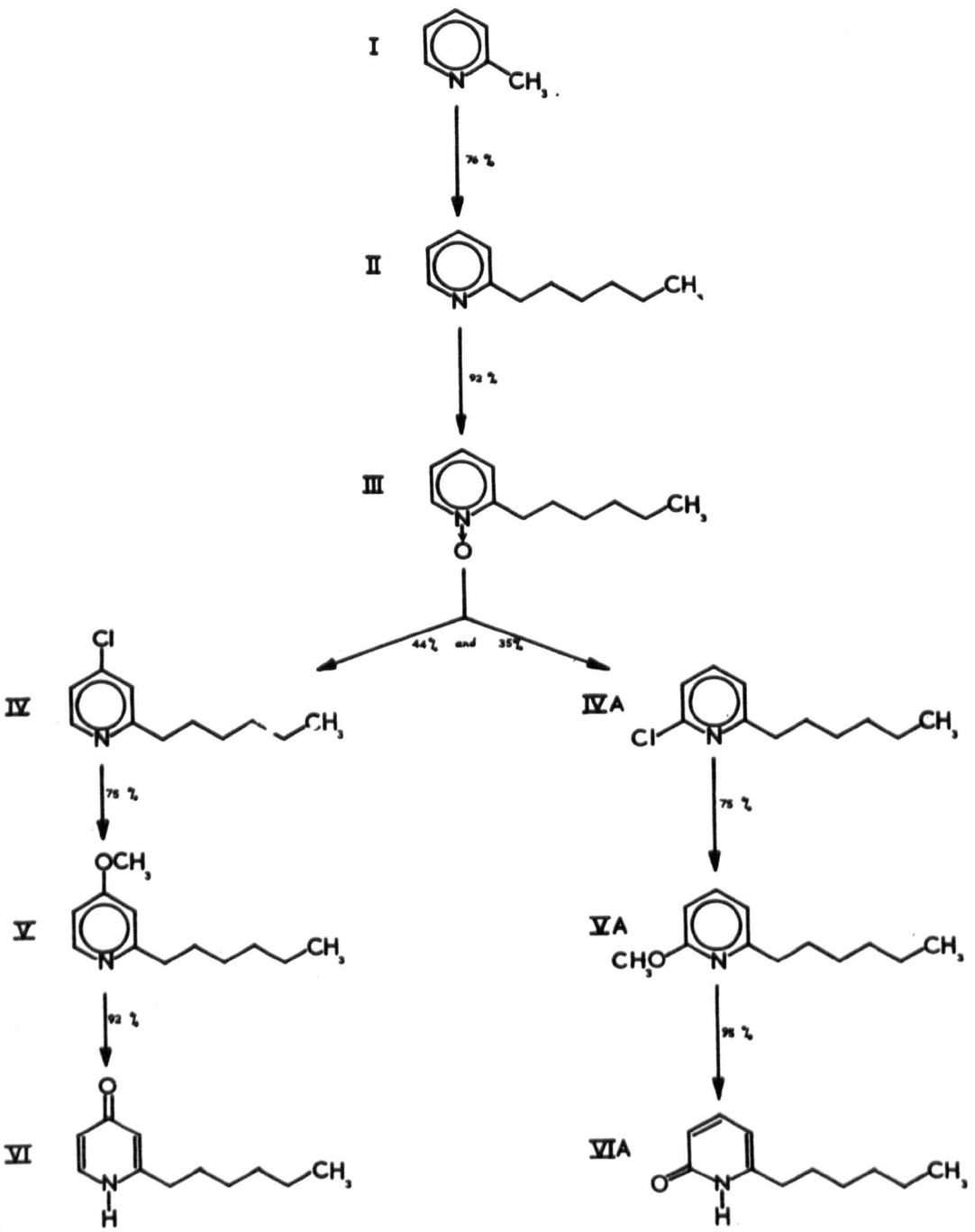
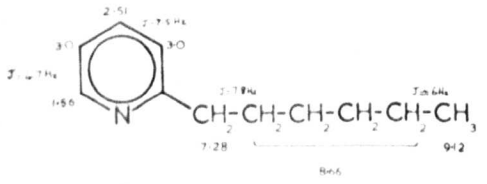


FIGURE 25

'H NMR ASSIGNMENTS

τ (CCl$_4$)



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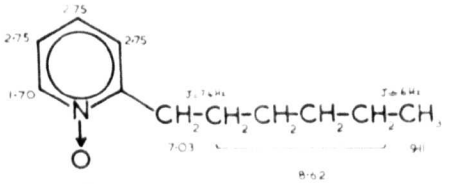

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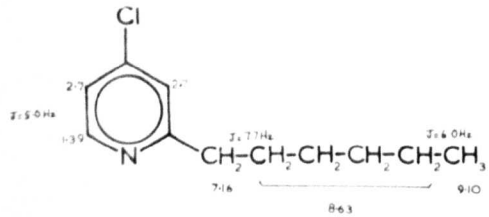
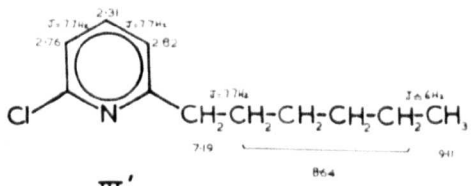
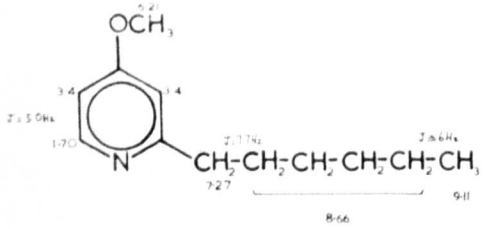


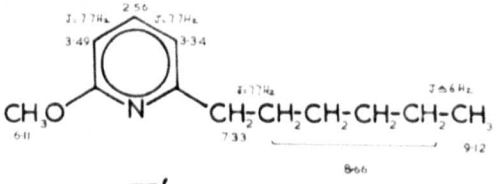
III



III'



IV



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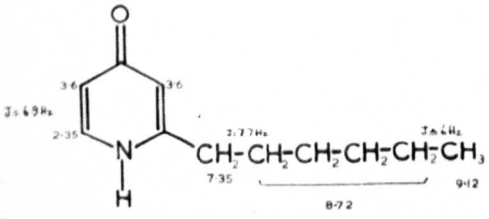
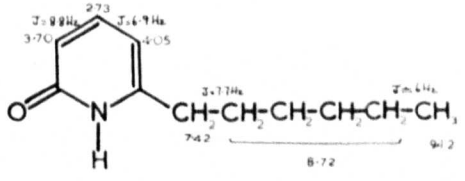


V



V'

THE SYNTHESIS OF COMPOUNDS TESTED FOR THEIR INHIBITION OF NADH-LINKED OXIDATION

Piericidin A has been shown to be a phenolic pyridine having a long hydrocarbon side-chain, a methyl group and two methoxyl groups as the remaining substituents of the pyridine ring[2]. The compounds whose synthesis is described below possess certain of these features, and were tested for their inhibition of NADH linked oxidation in mitochondria in comparison to piericidin A.

In addition, the hydroxypyridines amongst these compounds were used for the purpose of spectral comparisons pertaining to the piericidins described later (pages 95-121).

(i) The synthesis of 2-(1-n-hexyl)-4-hydroxypyridine and 6-(1-n-hexyl)-2-hydroxypyridine

Both compounds were synthesised in five stages from α-picoline as indicated in figure 24.

The reaction of α-picolyl lithium[184] with 1-bromopentane yielded 2-(1-n-hexyl)pyridine (II figure 24) as the sole isolated product (page 180), having UV and ^{1}H NMR spectra (interpreted in figure 25,I) reminiscent of α picoline (see figure 32). Its mass spectrum (see mass spectral appendix figure 42) is interpreted later (page 114), and is entirely consistent with the proposed structure.

In order to prepare the N-oxide of 2-(1-n-hexyl)pyridine it was necessary to use 35% peracetic acid as an oxidant, the *in situ* production of a dilute solution of this acid using hydrogen peroxide and acetic acid[169] affecting no reaction (page 181). Clearly the hexyl substituent is responsible for the difference (Cf page 196),

the most likely reason for this being that heterocyclic nitrogen atom is sterically less accessible to peracetic acid. Another possibility is that the ionisation of acetic acid, necessary to form peracetic acid by reaction with hydrogen peroxide, may be reduced by the presence of the long hydrocarbon substituent to such an extent that very little oxidant is present at any one time. The product obtained using 35% peracetic acid was identified as the desired N-Oxide, III (figure 24), by its strong IR absorption at 1245 cm^{-1} attributed to the N-O stretching vibration[185], and by its ^{1}H NMR spectrum which is interpreted in figure 25 (II).

All attempts to nitrate 2-(1-n-hexyl)pyridine-1-oxide failed, the original intention being to prepare a 4-hydroxy derivative via 2-(1-n-hexyl)-4-nitropyridine-1-oxide.

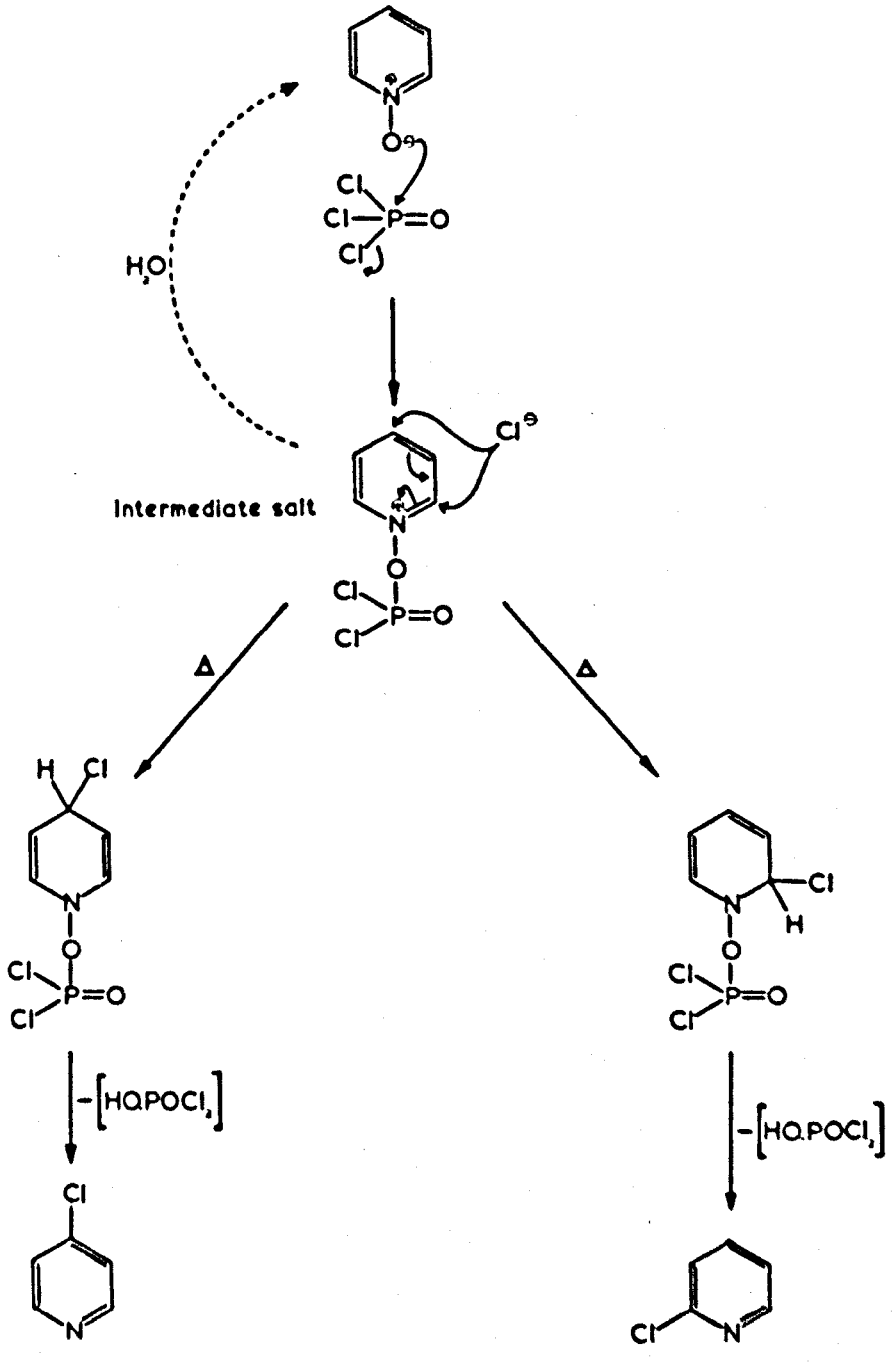
Using mixed acids under conditions where pyridine-1-oxides are normally nitrated[169] (pages 160,174,194,196), a considerable quantity of starting material was the only recovered material (page 181). The failure of the reaction can hardly be put down to steric factors in this case. A reduction ionisation of the acids, and hence of the concentration of nitronium ions, in the presence of the hexyl substituent might once again be the cause of failure. The use of nitronium tetrafluoroborate, a very hygroscopic but otherwise stable salt[186], appeared to offer a solution to this problem. Its use in the nitration of pyridine on the heterocyclic nitrogen atom, and of pyridinium fluoroborate on the β carbon atom has been reported[187]. By analogy, a nitration on carbon was attempted using the fluoroboric acid salt of 2-(1-n-hexyl)pyridine-1-oxide and nitronium tetra-

fluoroborate in acetonitrile or nitromethane at room temperature. Despite an exothermic reaction, in both solvents, only starting material was recovered after hydrolysis (page 182). It is conceivable that this exothermic reaction corresponds to a nitration on oxygen, which, as in the pyridine example[187b], effectively prevents any further nitration due to the withdrawal of electrons from the pyridine ring. Any O-nitro compound produced would of course be easily hydrolysed.

Reaction of 2-(1-n-hexyl)pyridine-1-oxide with phosphorus oxychloride yielded 4-chloro-2-(1-n-hexyl)pyridine and 6-chloro-2-(1-n-hexyl)pyridine as the only products (page 183). The reaction was analogous to that involving 2-methyl-pyridine-1-oxide[174b], the notable difference being that no compound corresponding to 2-chloromethylpyridine, which was isolated in that case, was found. It seems likely that such a compound would be formed by nucleophilic attack of a chloride ion on a methide intermediate (Cf figure 21, IIIc), and that its absence in the case of the reaction involving 2-(1-n-hexyl)pyridine-1-oxide implies that the loss of a proton required for the formation of a methide is less facile on account of the inductive effect of the alkyl substituent. Furthermore nucleophilic attack on such a methide would be sterically hindered. The two isomeric chloropyridines isolated were distinguished by the differences in the ^{1}H NMR signals of their aromatic protons. These ^{1}H NMR spectra are interpreted in figure 25 (III and III^1). The distinction was based on the following observations:

FIGURE 26

REACTION OF PYRIDINE-1-OXIDES WITH PHOSPHORUS OXYCHLORIDE



(i) α protons resonate at lower τ values than do β or γ protons in comparable pyridines[129], and pages 95-100

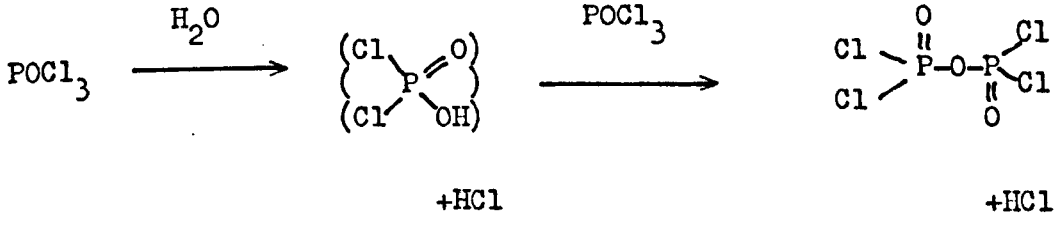
(ii) The spin-spin coupling constant between α and β protons in pyridines is typically in the region of 4½-5 Hz., whereas that between β and γ protons is around 8 Hz.[188].

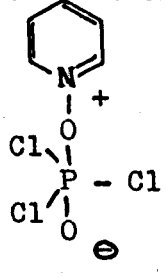
(iii) The triplet signal at 2.31 τ with J=7.7 Hz. can only be accounted for by the 2,6-disubstituted derivative.

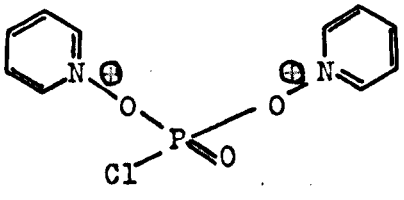
As mentioned earlier (page 75), it has been suggested that the initial step in the reaction between phosphorus oxychloride and pyridine-1-oxides is the formation of a salt, the chlorination occurring later (see figure 26).

The mixing of pyridine-1-oxides and phosphorus oxychloride at 0°C is exothermic (page 182), however the pyridine-1-oxide may be recovered unchanged on hydrolysis if the mixture is not heated (page 185). It therefore seems likely that the proposed salt was formed on mixing, since this is the only stage of the reaction which is likely to be reversible.

Some confirmation for this suspicion was gained by an examination of the ^{31}P NMR spectra of mixtures of pyridine-1-oxides with phosphorus oxychloride (page 185). A signal attributable to phosphorus oxychloride (from-2 to -4 ppm relative to that of 85% phosphoric acid) was only found when that material was present in an excess molar ratio. Two signals were found in all the mixtures. One, very weak, in the region of +8 to +10 ppm was attributed to pyrophosphoryl chloride[189] produced by traces of water.



The other signal, in the region of +3 to +4 p.p.m., was thought to be of the proposed salt (figure 26). The only other likely intermediate, having the structure  , would be expected to have a ^{31}P resonance at much higher field[190]. The small signal present when the pyridine-1-oxide was present in an excess molar ratio may be attributed to the following species:

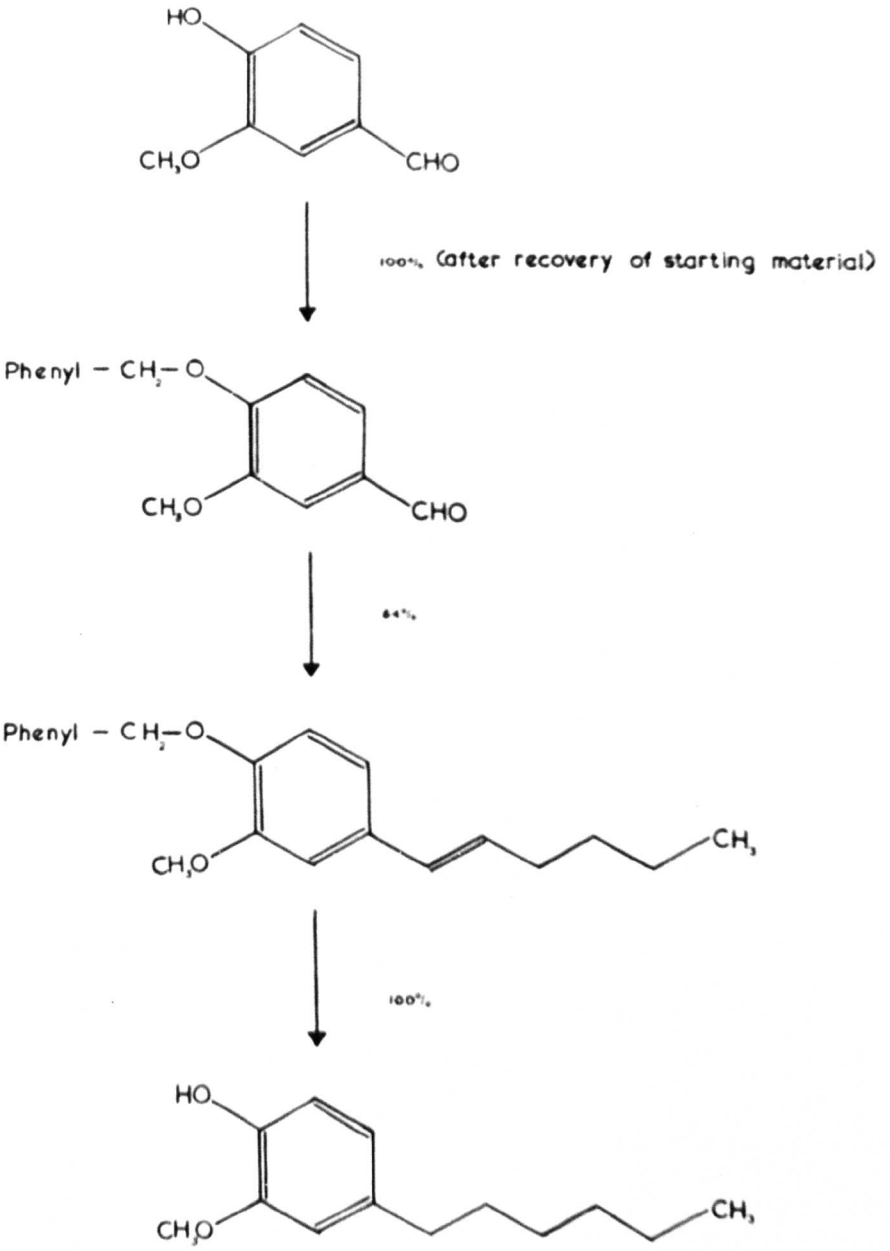


The above spectral results, together with the experiment mentioned earlier in which the pyridine-1-oxides were hydrolytically recovered, support the proposed mechanism of chlorination (figure 26), and indicate that the intermediate salt is quite stable in the absence of water.

The two chloropyridines, IV and IVA (figure 24), were converted to the corresponding methoxypyridines, V and VA, on treatment with sodium methoxide in methanol (pages 186-187) by analogy to published procedures[177]. The products were identified by their ^{1}H NMR spectra, which are interpreted in figure 25 (IV and IV[1]), and

FIGURE 27

SYNTHETIC ROUTE



their mass spectra (figure 42 of the mass spectral appendix, and page 114), and by the correspondence of their UV spectra to those of the unsubstituted 2-methoxypyridine and 4-methoxypyridine appropriately (see pages 210 - 211).

Acidic hydrolysis of these methoxypyridines[191] produced 2-(1-n-hexyl)-4-hydroxypyridine and 2-(1-n-hexyl)-6-hydroxypyridine, VI and VIA (figure 24) respectively, in high yields. These compounds were identified by their ^{1}H NMR spectra, which are interpreted in figure 25 (V and V^1), their mass spectra (figure 42 of the mass spectral appendix, and page 114), and by their elemental analyses (pages 187 - 188). Their strong IR absorptions at 1628 cm^{-1} (4-hydroxy derivative) and 1640 cm^{-1} (6-hydroxy derivative) are typical of 1-(H)-pyridone tautomers (page 37). Striking similarities of their UV spectra and parts of their ^{1}H NMR spectra with those of 2-hydroxypyridine and 4-hydroxypyridine as appropriate (page 210 - 211 and figure 32) confirm that the products exist predominantly as 1-(H)-pyridones and not as pyridinols. Such spectral comparisons are extended later (pages 95 - 121).

The yield of each stage in the two synthetic routes is shown in figure 24, overall yields for the five stage syntheses being 21% for 2-(1-n-hexyl)-4-hydroxypyridine and 17% for 2-(1-n-hexyl)-6-hydroxypyridine.

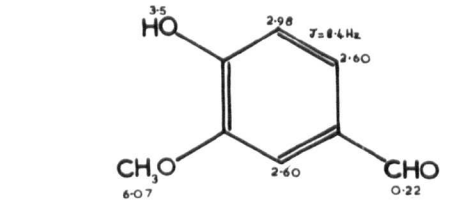
(ii) <u>The synthesis of 4-(1-n-hexyl)-2-methoxyphenol</u>

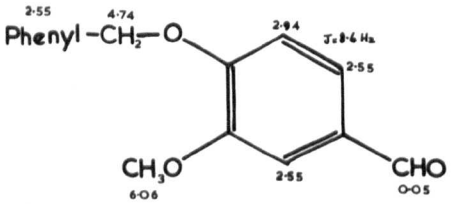
This compound was synthesised from vanillin in three stages as indicated in figure 27 (see pages 189 - 191).

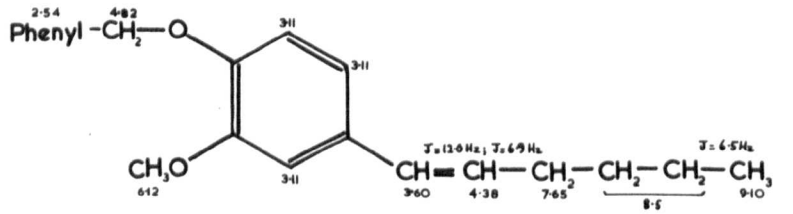
The phenolic function of vanillin was protected in the form of

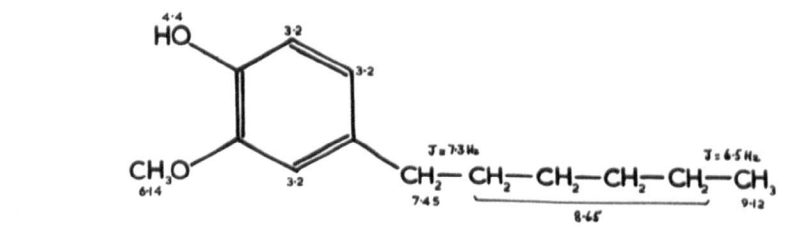
FIGURE 28

^{1}H NMR ASSIGNMENTS









its benzyl ether prepared via a Williamson ether synthesis[192]. A Wittig salt, formed from 1-bromopentane and triphenyl phosphine, was then employed in the alkylation of the benzyl vanillin to produce benzyl-(4-(1-n-hex-1-enyl)-2-methoxyphenyl)ether. Catalytic hydrogenation of this gave the desired 4-(1-n-hexyl)-2-methoxy-phenol.

All four compounds shown in figure 27 were chromatographically distinguishable, their reported structures being clearly indicated by their ^{1}H NMR spectra, which are interpreted in figure 28.

(iii) The synthesis of 4-hydroxy-2-hydroxymethyl-5-methoxy-pyridine

This compound was prepared by the successive methylation and ammonolysis of kojic acid according to standard procedures[193,194], (pages 192-193).

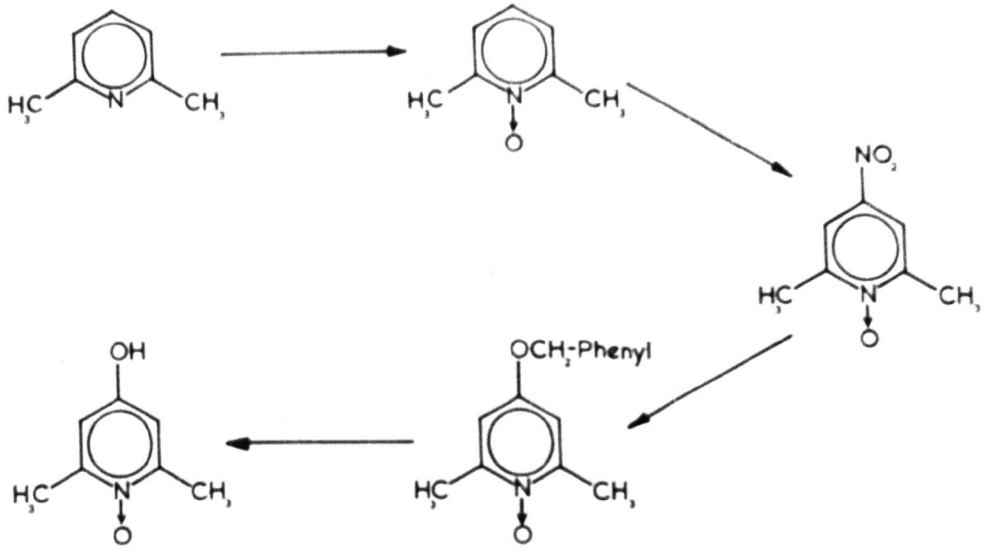
Its strong IR absorption at 1621 cm^{-1} is typical of a 1-(H)-4-pyridone (page 37), as is the behaviour of its UV absorption in acidic, neutral and basic solutions (pages 36 and 211). Its ^{1}H NMR spectrum is consistent with the proposed structure and also favours the 1-(H)-4-pyridone tautomer (see pages 95-101). An interpretation of the mass spectrum is given in figure 50 of the mass spectral appendix.

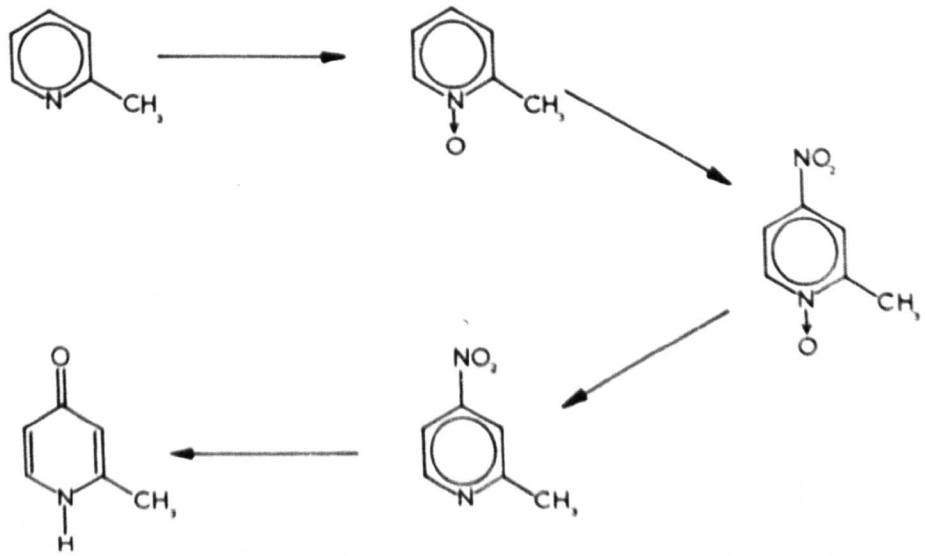
(iv) The synthesis of 2,6-dimethyl-4-hydroxypyridine-1-oxide

This compound was prepared in four stages from 2,6-lutidine as indicated in figure 29 (pages 194-195). Oxidation of the starting material with hydrogen peroxide in acetic acid, followed by nitration using mixed acids was according to the

FIGURE 29

SYNTHETIC ROUTES





standard procedures of Ochiai[169], yielding 2,6-dimethyl-4-nitropyridine-1-oxide. Nucleophilic displacement of the nitro group of this compound using sodium benzyloxide in benzyl alcohol was according to the method used for the same reaction on 4-nitropyridine-1-oxide, again by Ochiai[195]. Catalytic hydrogenation of 4-benzyloxy-2,6-dimethylpyridine-1-oxide, using 5% palladium on carbon in ethanol, gave 2,6-dimethyl-4-hydroxypyridine-1-oxide as the sole product. The fact that no 2,6-dimethyl-4-hydroxypyridine was isolated even after prolonged hydrogenation was not anticipated from the report of similar reactions employing these conditions[196]. It is concluded that the 2,6-disubstitution drastically retards the rate of reduction of the N-oxide function under these conditions, probably on account of steric hindrance of the same towards hydrogen bound to the catalyst.

(v) The synthesis of 4-hydroxy-2-methylpyridine

This compound was prepared in four stages from α-picoline as indicated in figure 29 (pages 196 - 197). 2-Methyl-4-nitropyridine was prepared according to the standard procedures of Ochiai[169] in three stages. This was converted to 4-hydroxy-2-methylpyridine by prolonged heating with water (page 196), in a manner similar to, but slower than, the preparation of 4-hydroxypyridine from 4-nitropyridine[197]. If the reaction involves nucleophilic displacement of the nitro group by water, then the electron donation of the additional alkyl substituent would be expected to reduce the rate of reaction.

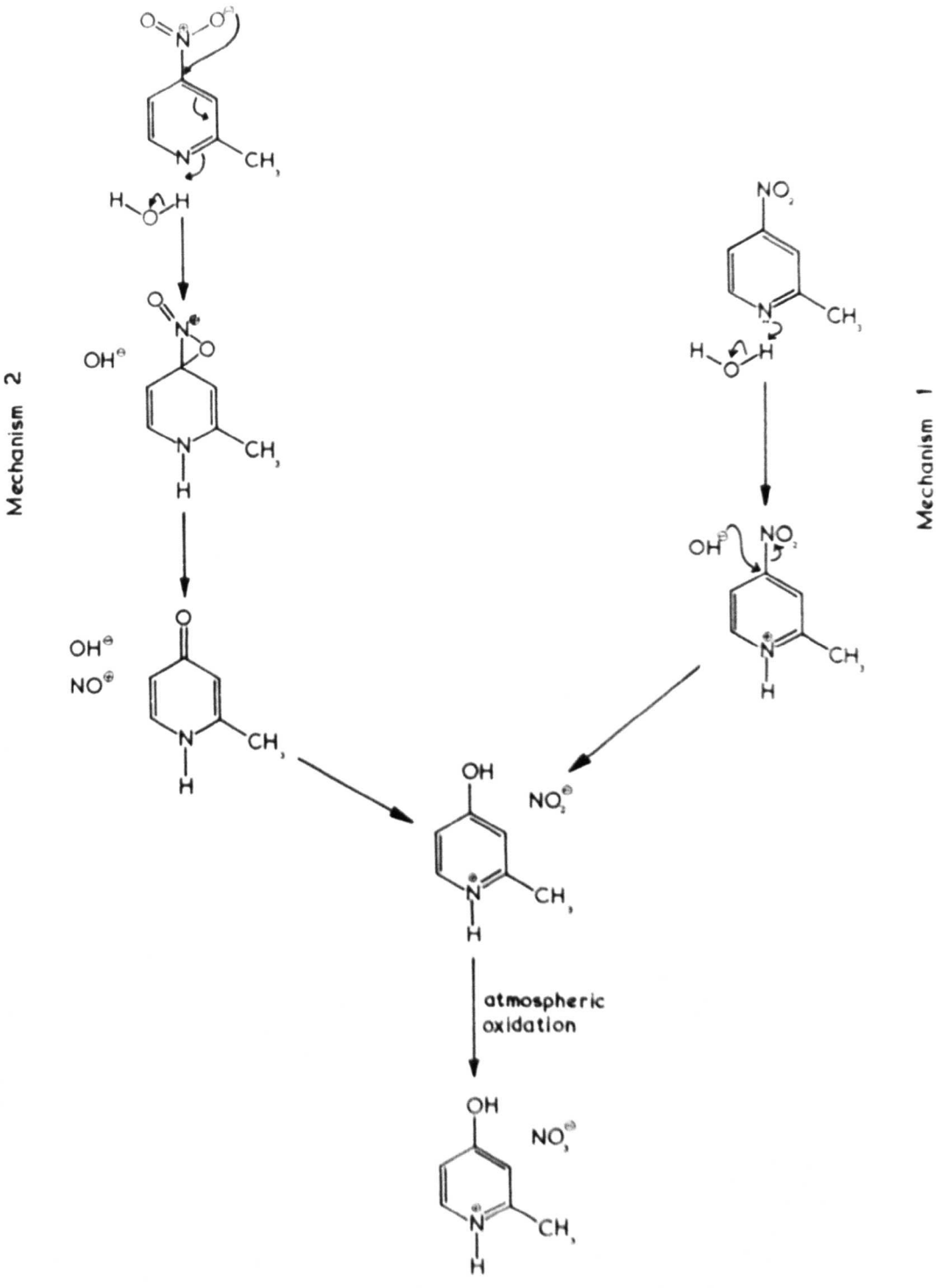
4-Hydroxy-2-methylpyridine was identified as a 1-(H)-pyridone from the changes of its UV spectrum in acidic, neutral and basic solution (Cf page 36), from its IR absorption at 1630 cm^{-1} (Cf page 37), and from its ^{1}H NMR spectrum (see pages 95-101).

It was discovered that 2-methyl-4-nitropyridine became converted to 4-hydroxy-2-methylpyridine nitrate in high yield on prolonged atmospheric exposure (page 197). The melting point of the product was in agreement with a reported value[198], but its identity was also assured by the chemical detection of nitrate ions and the existence of ions at m/e = 30 and 46 in the mass spectrum corresponding to NO^{+} and NO_2^{+} presumably derived from the nitrate moiety, and at m/e = 109, 94, and 80, corresponding to the molecular ion of 4-hydroxy-2-methylpyridine and ions derived from it by the loss of a methyl radical or of a formyl radical. Furthermore the ^{1}H NMR spectrum of the salt resembles that of 4-hydroxy-2-methylpyridine except that the resonance frequencies of the protons attached to carbon atoms are all found at lower field. This is only to be expected on protonation of the heterocycle[128].

The isolation of this salt in high yield, with none of the compound corresponding to 1-(4-pyridyl)-4-pyridone, reported in the case of the exposure of 4-nitropyridine to the atmosphere[197], is surprising. It is possible that the formation of the salt involves the nucleophilic displacement of the nitro group by atmospheric water, the nitrite salt so formed being afterwards oxidised to a nitrate by atmospheric oxygen (Mechanism 1, figure 30). However the possibility of an intramolecular rearrangement of the nitro

FIGURE 30

TWO DIFFERENT MECHANISMS OF REACTION



group, proposed in a similar example[199] is equally feasible (Mechanism 2, figure 30). The distinctive difference lies in the source of oxygen present in the pyridine moiety of the product.

THE SYNTHESIS OF OTHER PYRIDINES AND EXPERIMENTS CONCERNED WITH THE SYNTHESIS OF PYRIDINES

3-Methoxypyridine and 5-methoxy-2-methylpyridine were prepared by methylation of the corresponding hydroxypyridines with diazomethane in homogeneous solution (pages 199-200).

4-Methoxypyridine was prepared from 4-chloropyridine by the action of sodium methoxide according to a standard procedure[200], (page 200).

1-methyl-4-pyridone was prepared by methylation of the potassium salt of 4-hydroxypyride with methyl iodide, the method being derived from a similar alkylation of 4-hydroxypyridine[201], (page 200)

1-methyl-2-pyridone was prepared by the thermal isomerisation of 2-methoxypyridine at 230°C, there being no conversion at 180°C, and was a solid at room temperature.

The UV, IR, ^{1}H NMR and mass spectra of the above compounds are considered later in comparisons with related pyridines (pages 95-121). They are all consistent with the proposed structures.

The facile substitution of hydroxyl or methoxyl groups into the pyridine nucleus is clearly a subject of interest in the preparation of compounds related to the piericidins. Nucleophilic substitution reactions of this type are well known[202a], some of them being employed in syntheses described in this thesis (pages 76,80,85,90 and above). On the other hand comparatively little is known about the electrophilic oxygenation of pyridines[202b, 203]. Elbs

oxidation has been employed in the preparation of 2,5-dihydroxypyridine[204], but the reagent has the disadvantage of being a powerful oxidant. Aromatic hydroxylations with Fenton's reagent and other reagents involving hydroxyl radicals which are characteristically electrophilic, are established in other systems[205]. The slow oxidation of pyridine to 3-hydroxypyridine, which was reported some years ago[206], using hydrogen peroxide as the sole reagent at 50°C, may be a reaction of this type. This experiment was repeated (page 202), but no 3-hydroxypyridine or even pyridine-1-oxide, was isolated. The somewhat unstable solid produced, which was water soluble and oxidised starch/iodide paper, may have been a peroxide salt.

The mild hydroxylation of nicotine involving enzymic hydration followed by oxidation, reminiscent of the older Decker oxidation of 1-methylpyridinium iodide to 1-methyl-2-pyridone[207], does not appear to offer general applicability to other pyridines[208].

It is concluded that the direct introduction of hydroxyl or alkoxyl groups into pyridine nuclei is usually most conveniently achieved by nucleophilic substitution.

FIGURE 31

^{1}H NMR ASSIGNMENTS

τ ($CDCl_3$)

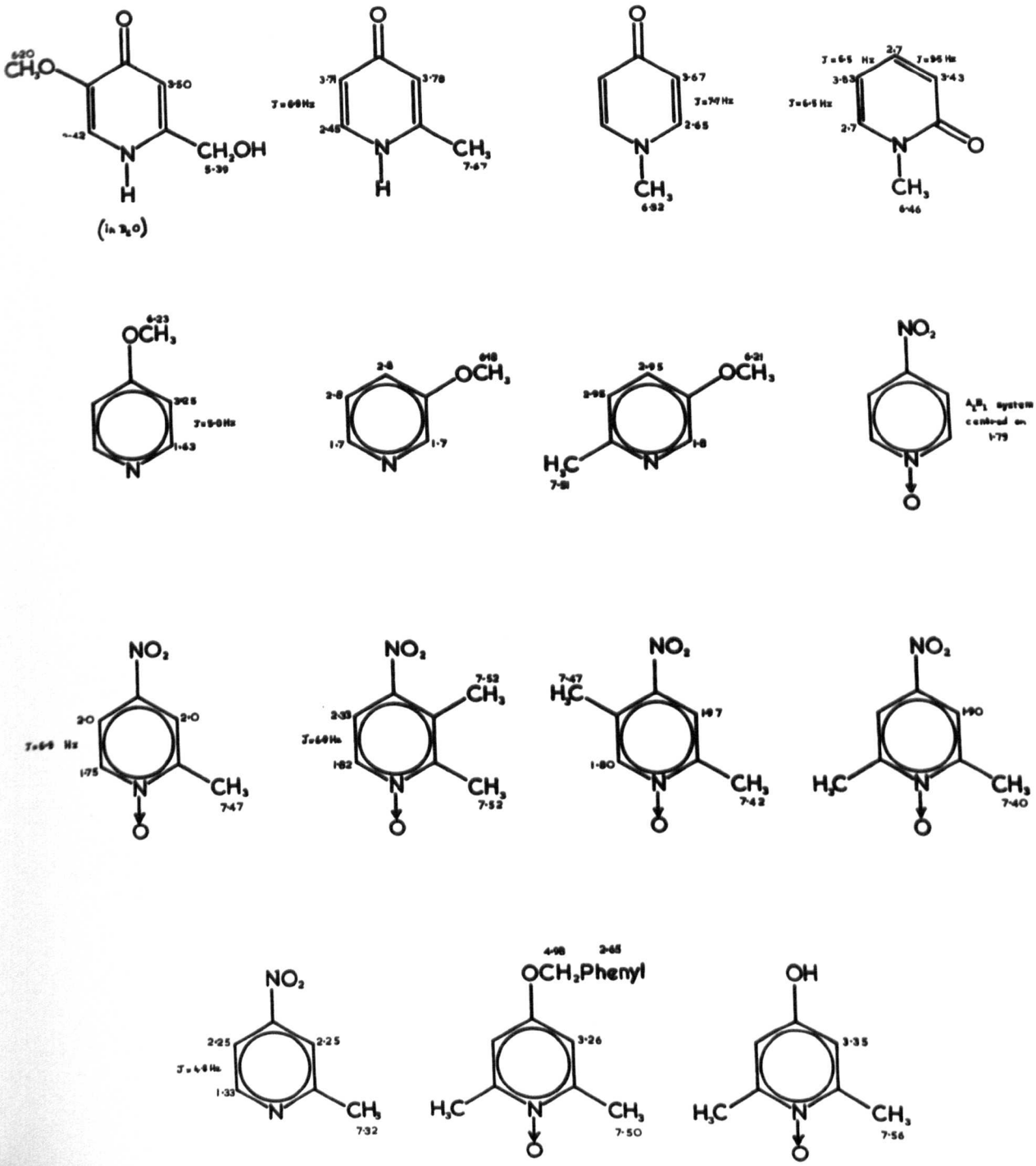
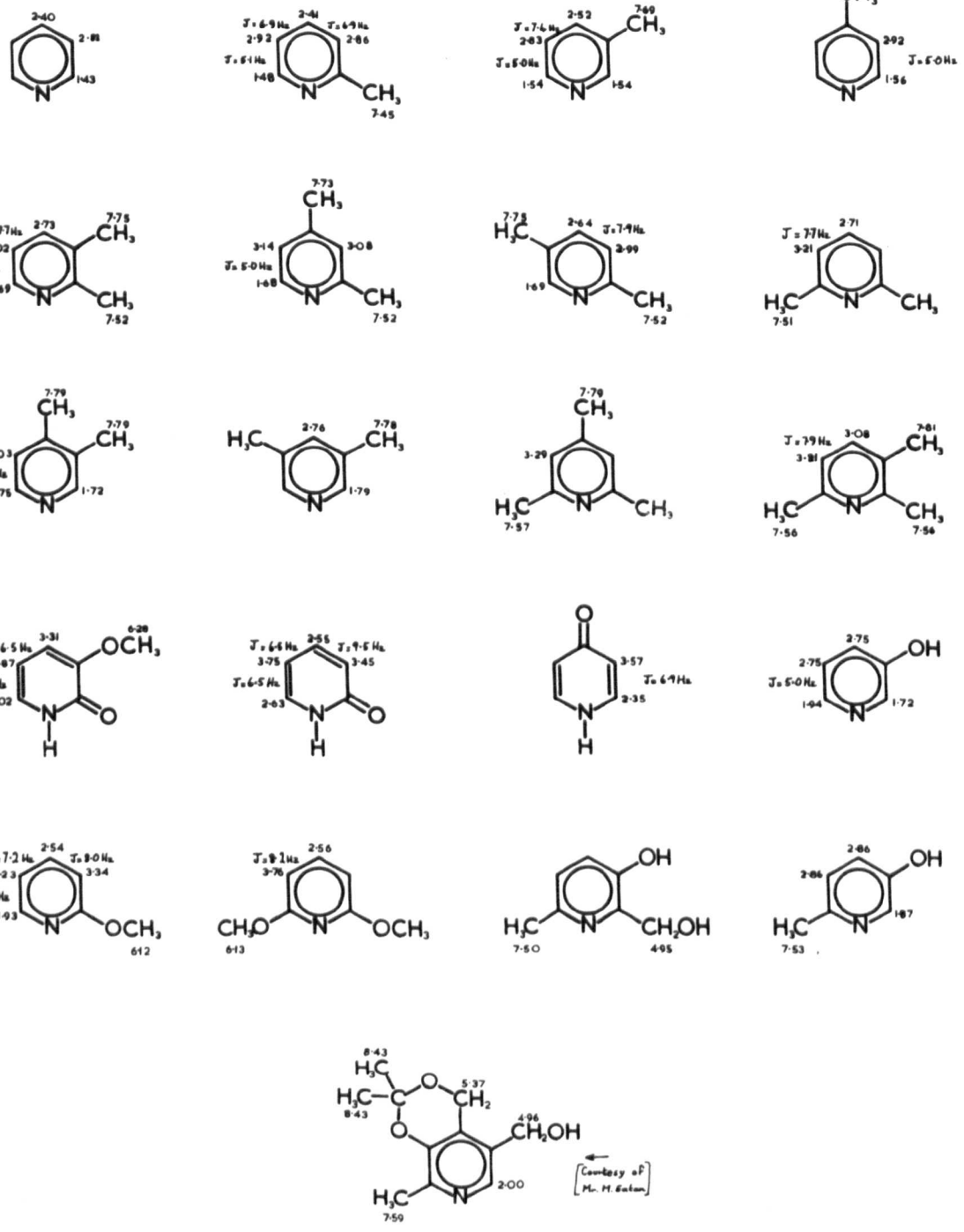


FIGURE 32

^{1}H NMR ASSIGNMENTS

τ ($CDCl_3$)



EXPERIMENTAL COMPARISONS INVOLVING SUBSTITUENTS IN THE α, β and ɣ POSITIONS OF PYRIDINES

(i) The interpretation of ^{1}H nuclear magnetic resonance spectra

During the course of work described in this thesis the ^{1}H NMR spectra of a number of pyridine compounds having alkyl, methoxyl and hydroxyl (or tautomeric hydroxyl) functions were recorded. These spectra are interpreted in figures 23, 25 and 31. Additionally the spectra of a number of similar readily available pyridines were recorded (pages 203-204). These are interpreted in figure 32. All the spectra were of 10% w/v solutions of the compounds in deuterochloroform whenever possible.

Careful comparisons of all these revealed a number of similarities by which a degree of predictability may be conferred towards the spectra of any other pyridine bearing exclusively such substituents.

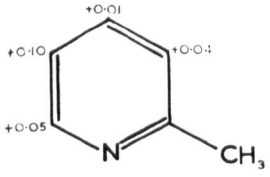
(a) Chemical Shifts

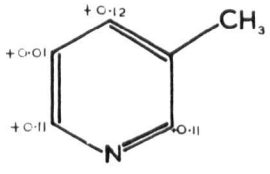
There were differences in the chemical shifts of nuclear protons in the spectra of 2,3- and 4-methyl-, methoxy- and hydroxy-pyridines compared to those in the spectrum of pyridine itself. These are summarised in figure 33. The comparatively greater changes in the cases of 2- and 4-hydroxypyridine are accounted for by the predominance of pyridone structures in these compounds. In all nine compounds the τ values of α protons, and of all protons ortho or para to the additional substituent, were greater than in pyridine itself, presumably due in part to greater local electron density (i.e. shielding) around these protons.

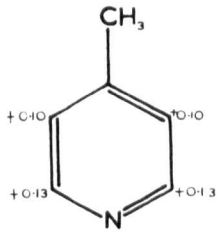
FIGURE 33

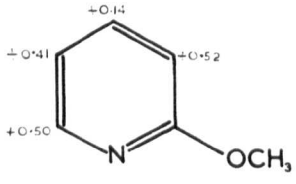
DIFFERENCES OF CHEMICAL SHIFT COMPARED TO THE PROTONS OF PYRIDINE

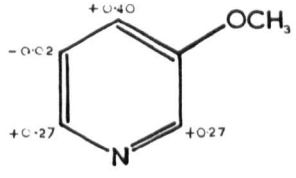
(expressed as τ value differences)

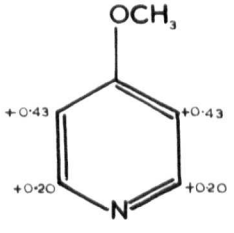


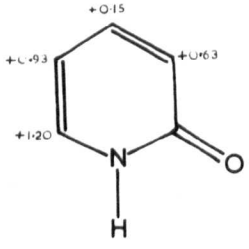


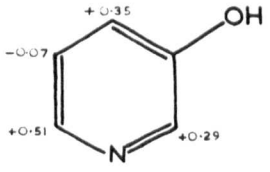


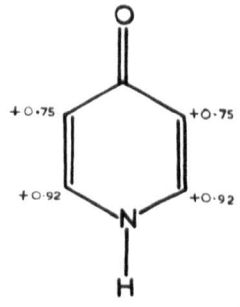












β or γ protons which were meta to the additional substituent showed variable shifts much smaller than those observed elsewhere in the particular molecule. This may be accounted for by the absence of any mesomeric effect due to the additional substituent on these protons, and their consequent dependence on the inductive effect of the same. The fact that protons meta to the additional substituent but in an α position behaved differently indicates that they are considerably affected by the electron attracting demands of the heterocyclic nitrogen atom. The presence of an electron donating substituent elsewhere in the molecule may partially satisfy these demands, and in so doing reduce the attraction of electrons from the rest of the nucleus in general, the effect being particularly noticeable in the α position.

The chemical shifts of nuclear protons in more highly substituted pyridines containing methyl, methoxyl and hydroxyl substituents, or their equivalents, can be estimated from the data in figure 33. The sum of the differences of chemical shift shown in figure 33, of relevence to a particular nuclear proton, attributable to each additional substituent considered in isolation, gives a moderately reliable estimate of the actual difference between the observed chemical shift of the proton and that of the equivalent proton in pyridine itself. Two examples of this calculation are shown below, the results of others being listed in Table 2. It can be seen that the estimates are frequently, but not always, slightly too small.

Calculation of the chemical shift of the 5-proton in 4,6-dimethoxy-2,3-dimethyl pyridine

	τ
Chemical shift of β proton in pyridine	+ 2.82
Change in chemical shift of 5-proton due to 2-methyl group	+ 0.10
" " " " " 5- " " " 3-methyl group	+ 0.01
" " " " " 5- " " " 4-methoxyl group	+ 0.43
" " " " " 5- " " 6-methoxyl group	+ 0.52
Predicted chemical shift of 5-proton	+ 3.88
Observed value (page 165)	+ 3.97

Calculation of the chemical shift of the 3-proton in 4,6-dimethoxy-2,5-dimethylpyridine

	τ
Chemical shift of β-proton in pyridine	+ 2.82
Change in chemical shift of 5-proton due to 2-methyl group	+ 0.04
" " " " " 5- " " " 4-methoxyl group	+ 0.43
" " " " " 5- " " " 5-methyl group	+ 0.01
" " " " " 5- " " " 6-methoxyl group	+ 0.41
Predicted chemical shift of 5-proton	+ 3.71
Observed value (page 175)	+ 3.67

No estimations of the type just mentioned could be formulated to predict the chemical shift of protons in methyl and methoxyl substituents in pyridines. However it was noted that the resonance frequency of protons in α-methyl groups was usually of the order of 7.5 to 7.7 τ, whereas in β or γ-methyl groups it was typically between 7.7 and 7.9 τ

TABLE 2

PREDICTION OF THE CHEMICAL SHIFTS OF NUCLEAR PROTONS IN PYRIDINES BEARING ALKYL, ALKOXYL AND HYDROXYL (OR TAUTOMERIC HYDROXYL) FUNCTIONS EXCLUSIVELY

The observed τ values of the nuclear protons of each molecule, numbering the positions of substitution 2-6 in a clockwise direction, are listed. Under each of these values, the figure in brackets is that of the predicted τ value of the proton.

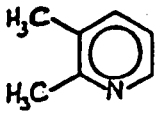
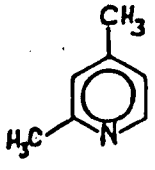
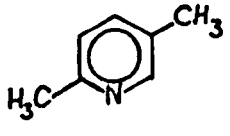
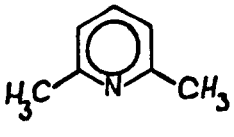
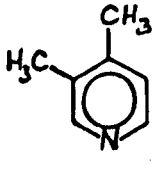
Compound	POSITION OF SUBSTITUTION OF NUCLEAR PROTON				
	2	3	4	5	6
			2.73 (2.53)	3.02 (2.93)	1.69 (1.59)
		3.08 (2.96)		3.14 (3.02)	1.68 (1.61)
		2.99 (2.87)	2.64 (2.53)		1.69 (1.59)
		3.21 (2.96)	2.71 (2.42)	3.21 (2.96)	
	1.72 (1.67)			3.03 (2.93)	1.75 (1.67)

TABLE 2 cont'd

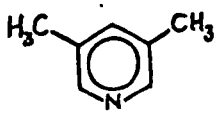
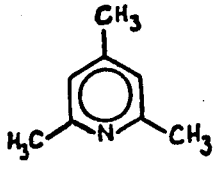
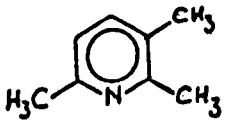
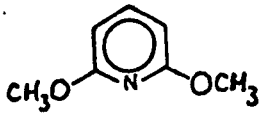
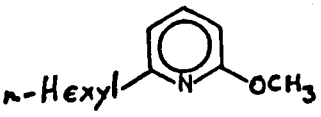
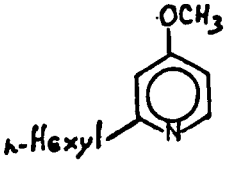
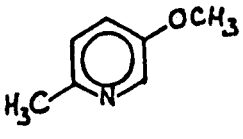
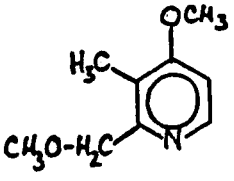
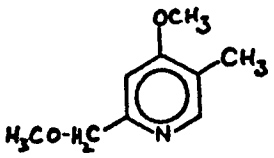
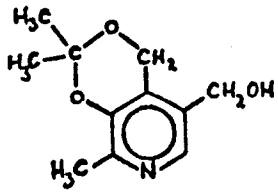
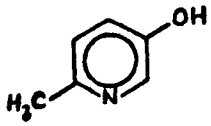
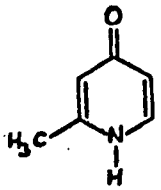
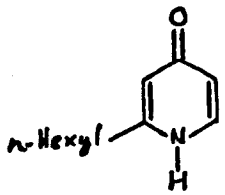
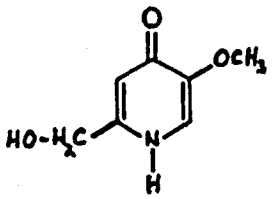
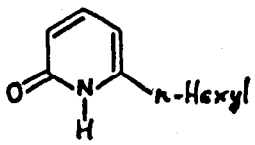
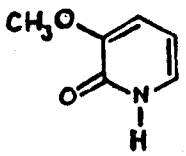
Compound	2	3	4	5	6
	1.79 (1.65)		2.76 (2.64)		1.79 (1.65)
		3.29 (3.06)		3.29 (3.06)	
		3.21 (2.97)	3.08 (2.54)		
		3.76 (3.75)	2.56 (2.68)	3.76 (3.75)	
		3.34 (3.27)	2.56 (2.55)	3.49 (3.44)	
		3.4 (3.29)		3.4 (3.35)	1.70 (1.68)
		2.95 (2.80)	2.95 (2.81)		1.8 (1.75)
				3.37 (3.36)	1.72 (1.79)

TABLE 2 cont'd

Compound	2	3	4	5	6
		3.13 (3.30)			1.86 (1.79)
					2.00 (1.99)
		2.86 (2.76)	2.86 (2.76)		1.87 (1.77)
		3.78 (3.61)		3.71 (3.67)	2.45 (2.40)
		3.6 (3.61)		3.6 (3.67)	2.35 (2.40)
		3.50 in D_2O (3.59)			2.42 in D_2O (2.67)
		3.70 (3.55)	2.73 (2.56)	4.05 (3.79)	
			3.31 (2.95)	3.87 (3.73)	3.02 (2.90)

Similarly the resonance frequency of protons in α-methoxyl groups was usually found near 6.1 τ, whereas in β-or γ-methoxyl groups a value close to 6.2 τ was typical. A greater number of alkyl, alkoxyl or hydroxyl functions in the pyridine nucleus usually resulted in higher τ values for the resonance frequency of protons in a particular methyl group, but the effect on the resonance frequency of protons in methoxyl groups was variable. Finally the τ values of the absorptions of protons in methyl and methoxyl substituents attached to pyridones were often higher than might be expected from the above remarks. This is accounted for by the reduced aromatic ring-current effect likely in these compounds.

The τ values of the two methoxyl substituents and of the methyl substituent of the pyridine ring in piericidin A are 6.08 τ, 6.18 τ and 7.91 τ respectively. It is deduced from the above considerations that one of the methoxyl substituents is in an α - position in the pyridine ring, whilst the other methoxyl substituent and the methyl substituent are each in either a β- or a γ-position. It has been deduced independently (page 108) that the hydroxyl group of the pyridine nucleus of piericidin A is <u>not</u> in an α-position, and <u>ipso facto</u> it must be in a β-or γ-position. It therefore follows that the long side-chain of piericidin A is in an α-position in the pyridine ring. The same conclusion is arrived at from mass spectral studies (pages 45, 57, 121).

Thus the contention on page 51 that piericidin A is a pyridine compound, substituted in the α-positions with a methoxyl group and a long hydrocarbon side-chain, and in the β- and γ-positions

with a methyl group, a methoxyl group and a hydroxyl group in an unspecified manner, is completely in line with its ^1H NMR spectrum.

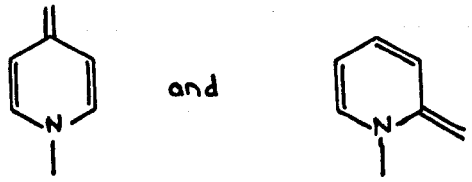
(b) Coupling constants

The spin-spin coupling constants between nuclear protons in pyridines are reported to be in the region of 4½-5 Hz between β and α protons[188]. These observations agree well with most of the values recorded in this thesis. However the cases of 2- and 4-hydroxypyridines and of 4-nitropyridine-1-oxides appear to be significant exceptions. Their coupling constants are listed below.

4-hydroxypyridine	$J_{\alpha\beta}$	6.9 Hz
4-hydroxy-2-methylpyridine	$J_{\alpha\beta}$	6.9 Hz
2-(1-n-hexyl)-4-hydroxypyridine	$J_{\alpha\beta}$	6.9 Hz
1-methyl-4-pyridone	$J_{\alpha\beta}$	7.7 Hz
2-hydroxypyridine	$J_{\alpha\beta}$	6.5 Hz
	$J_{\beta\gamma}$	6.5 Hz
	$J_{\beta 1\gamma}$	8.8 Hz
6-(1-n-hexyl)2-hydroxy-pyridine	$J_{\beta\gamma}$	6.9 Hz
	$J_{\beta}^{1}{}_{\gamma}$	8.8 Hz
2-hydroxy-3-methoxy-pyridine	$J_{\alpha\beta}$	6.5 Hz
	$J_{\beta\gamma}$	6.5 Hz
1-methyl-2-pyridone	$J_{\alpha\beta}$	6.5 Hz
	$J_{\beta\gamma}$	6.5 Hz
	$J_{\beta}^{1}{}_{\gamma}$	9.5 Hz

4-nitropyridine-1-oxide	$J_{\alpha\beta}$ 8.8 Hz (A_2B_2 system)
2-methyl-4-nitropyridine-1-oxide	$J_{\alpha\beta}$ 6.9 Hz
2,3-dimethyl-4-nitropyridine-1-oxide	$J_{\alpha\beta}$ 6.9 Hz

The values of these coupling constants can be attributed to the predominance of structures of the type



in these compounds, rather than the more common form of the pyridine nucleus represented by

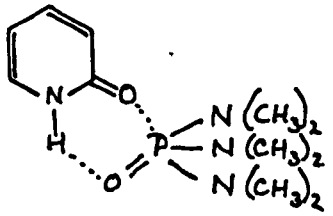
(ii) <u>^{1}H nuclear magnetic resonance spectra in hexamethylphosphoramide</u>

The use of hexamethylphosphoramide (HMPA) in the examination of mixtures of phenols has been reported recently[209]. In this solvent the resonances of phenolic protons, which in many solvents are normally broad, are considerably sharpened and usually appear in the region from $-0.3\,\tau$ to $+1.1\,\tau$, conveniently apart from aromatic protons. Within this range a distinction has been drawn between various types of alkylated phenols. Moreover, electron-withdrawing substituents were seen to produce a lowering of the τ value of the phenolic signal, the effect of electron-donating

substituents being variable. The signals were independent of concentration in the range of molarity from 0.05 to 1.0.

These studies have been extended in the hope of finding a facile distinction between 2-,3- and 4-hydroxypyridines (pages 204-205).

The phenolic (or amidic) signals of 2-hydroxypyridine, 2- and 4-hydroxyquinoline and 2-hydroxy-1-napthaldehyde were all below $-2\,\tau$, probably indicating a strong interaction with the solvent of the type shown below.



Uracil, which exhibited a doublet at -1.6 and $-1.7\,\tau$, may interact similarly with two solvent molecules.

The remaining examples are best considered in two groups; firstly those having a phenolic signal with the breadth at half height less than 30 Hz, and secondly those with very broad flat phenolic signals.

The resonances of phenolic protons in the first group were all in the region from $-1\,\tau$ to $0\,\tau$. Little distinction apparently can be made between 3- and 4-hydroxypyridines and other phenols of this group.

The breadth of the signals in the second group seems attributable to more than one factor. The suspicion that the phenolic groups of 4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine and its isomer might be sterically hindered from interaction with the solvent was supported by the equally broad signal found in the case of 2,6-di-t-butyl-4-

methylphenol. The line broadening observed in the cases of 4-hydroxy-2-hydroxymethyl-5-methoxypyridine, 2,3-dihydroxypyridine and 8-hydroxyquinoline is of a less obvious origin. Possibly it is evidence of faster chemical exchange caused by the interaction of the solvent at alternate localities of the molecules.

It is thus concluded that HMPA cannot be used as a solvent to distinguish between 3- and 4-hydroxypyridines by ^{1}H NMR spectroscopy, but that it may interact with 2-hydroxypyridines to produce a characteristic signal below -2 τ . In the case of hydroxypyridines in which the phenolic group is sterically hindered from interaction with the solvent, a broad ^{1}H NMR signal of no value in characterisation is likely to be produced. The use of HMPA as a solvent for the characterisation of the phenolic pyridine nucleus of piericidin A by ^{1}H NMR spectroscopy is therefore valueless.

Interactions of HMPA with alcohol, amine and amide functions were observed in the course of this investigation.

The signal at + 2.5 τ in the spectrum of 1-hydroxymethyl-2-naphthol was attributed to the alcoholic proton of this molecule on comparison with the spectrum of 1-methyl-2-naphthol.

The amino protons of aniline were found at 4.6 τ as a singlet.

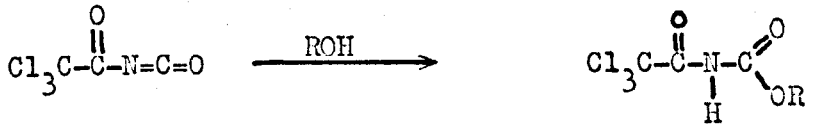
Two equally sized singlets, equivalent to one proton each, were found in the spectra of acetamide and benzamide at 1.95 and 2.95 τ , and at 1.14 and 2.25 τ respectively. Presumably the lower field signal in each case corresponds to an amidic proton involved in a solvent interaction of the type mentioned earlier, whereas the higher field signal corresponds to the other amidic proton which is relatively

uninvolved in such interactions. The slow rates of isotopic exchange observed in the cases of these latter amidic protons is in line with the weak basicity of amides compared to amines.

These latter results indicate that HMPA may be used as a solvent for ^{1}H NMR spectroscopy, to distinguish between phenols, alcohols, amides and amines.

(iii) The use of trichloroacetylisocyanate in ^{1}H nuclear magnetic resonance spectroscopy

The *in situ* reaction of trichloroacetylisocyanate (TAI) with alcohols and phenols for ^{1}H NMR studies has been described recently[210,211]. The product of reaction, the carbamate (II), was found to exhibit an N-H signal within the range -1 to + 2 τ.



TAI (I) II

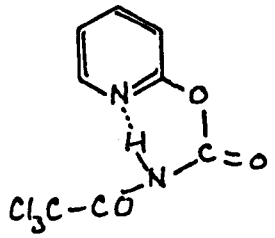
In the case of primary and secondary alcohols, the ^{1}H NMR signals of protons α to the alcoholic function shifted appreciably downfield on reaction, typical of their behaviour on esterification[212]. No such effects on the substituents of phenols after reaction are reported.

These studies have been extended in the hope of discovering a facile distinction between 2-,3-and 4-hydroxypyridines (pages 205-209 and Table 13.) The solvent of choice was deuterochloroform for the reasons described on page 206.

(a) The carbamate N-H signal

All the α-hydroxypyridines examined exhibited carbamate

N-H signals between -4 and -5 τ. Signals at such a low field imply hydrogen-bonding of the carbamate as indicated below.



The corresponding signals of all the 4-hydroxypyridines examined, and of 4-hydroxyquinoline, occurred between -2.3 and + 0.8 τ, whereas those of the β-hydroxypyridines examined were within the much smaller range from +0.4 to + 1.1 τ, with the exception of 3-hydroxypyridine itself in which the resonance was at -1.1 τ.

Of the remaining compounds, 8-hydroxyquinoline produced a carbamate N-H signal at -0.1 τ, typical of a phenol. 2-Hydroxyquinoline, which was only sparingly soluble in chloroform, did not appear to react at all, possibly on account of strong intermolecular hydrogen-bonding. Uracil and 2,6-dihydroxy-4-methylpyrimidine were completely insoluble in chloroform and also gave no detectable sign of reaction.

Signals attributable to reaction of the alcoholic functions of 3-hydroxy-2-hydroxymethyl-6-methylpyridine and of 4-hydroxy-2-hydroxymethyl-5-methoxypyridine were found at + 1.1 and + 3.3 τ respectively. Two distinct carbamate N-H signals were found in all the cases where dihydroxy-compounds reacted with TAI.

It appears from the above results that 2-hydroxypyridines are clearly distinguishable from 3- and 4-hydroxypyridines using TAI. The distinction between 3- and 4-hydroxypyridines is less clearcut, but generally the carbamate N-H signals of 4-hydroxypyridines are

found at lower field than those of 3-hydroxypyridines after reaction with TAI.

The corresponding signals derived from alcoholic functions appear to be at higher field than those derived from any of the hydroxypyridines.

From the results obtained with 4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine (carbamate N-H signal at + 1.06 τ) and with 4,6-dimethoxy-2,5-dimethyl-3-hydroxypyridine (carbamate signal at 1.01 τ), it is concluded that the presence of electron-donating substituents in a phenol raises the carbamate NH signal to higher field than would otherwise be the case.

Piericidins A and B both react with TAI (see figure 36, and pages 132-134) . The alcoholic carbamate NH signal of piericidin A appears at 1.77 τ whilst in both cases the phenolic carbamate NH signal is between 0.9 and 1.0 τ, very close to those of the isomeric dimethoxydimethylhydroxypyridines mentioned above. The obvious inference is that, although the pyridine nucleus of the piericidins does not correspond to either of these isomers, it is very closely related to them, and may well be a β-hydroxypyridine. It can be confidently asserted that piericidins A and B are not α-hydroxypyridines.

The signal at 6.36 τ (doublet, J = 8.9 Hz.) in the ^{1}H NMR spectrum of piericidin A is attributed to the proton α to the alcoholic function. After reaction with TAI the resonance of this proton is found at 4.99 τ (doublet, J = 8.9 Hz.) (page 132, figure 36) in accordance with the observations mentioned earlier[210-211] (page 106).

Similarly in the case of piericidin A diacetate the corresponding signal is found at 5.06 τ (doublet, J = 8.9 Hz) as expected[212] (see figure 36 and pages 56, 137). The comparable signals in the spectrum of piericidin B are found at 6.7 and 6.6 τ, before and after reaction with TAI (see figures 35 and 36 and page 134). These results confirm that piericidin A possesses a secondary alcohol function which is methylated in piericidin B.

(b) The chemical shift of substituents in hydroxypyridines

After reaction with TAI the ^{1}H NMR signals attributable to the substituents of a particular hydroxypyridine were usually observed at lower field (see Table 13). The magnitude of this change was highly variable, showing some dependence on the position of the substituent relative to the heterocyclic nitrogen atom and to the phenolic group, as well on the nature of other substituents.

The biggest change of chemical shift was frequently observed for a substituent in an α-position in the heterocycle. (Compounds 1, 2, 3, 4, 8, 10, 11 in Table 13). On the other hand in the cases of 4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine and its isomer upfield shifts were observed in the ^{1}H NMR signals of α substituents adjacent to the reacting phenolic group.

Upfield shifts were also observed in the signal of the heterocyclic methyl group of piericidins A and B after reaction with TAI. No conclusion was drawn from this observation on account of the diversity and lack of predictability apparent from the other examples.

With many of the hydroxypyridines whose spectra were recorded, alternative reaction of TAI at either oxygen or nitrogen would seem

possible. An example was mentioned earlier (page 78) in which two products resulted from reaction with TAI, although in that case reaction appeared to be at alternative oxygen atoms. Since without exception only one product is apparent from the ^{1}H NMR spectra of the above hydroxypyridines after reaction with TAI, it is assumed that this is formed by reaction at the oxygen atom, in accord with general observations on the acylation of hydroxypyridines[147].

(iv) Comparisons of infra-red spectra

The distinction between pyridinols and 1-(H)-pyridones mentioned earlier (page 37) was borne out by the IR spectra of seven further hydroxypyridines.

4,6-Dimethoxy-2,3-dimethyl-5-hydroxypyridine (page 172), 4,6-dimethoxy-2,5-dimethyl-3-hydroxypyridine (page 179), and 2,4-dimethyl-3-hydroxy-6-methoxypyridine[213] exhibited strong, sharp absorptions in solution at 3550, 3550 and 3610 cm^{-1} respectively, typical of the O-H stretching vibrations of free hydroxyl groups in pyridinols (page 37). In addition each compound absorbed strongly at 1610, 1609 and 1615 cm^{-1} respectively. These absorptions are attributed to ring vibrations although they are a little higher than is usual in pyridines[214].

In contrast 4-hydroxy-2-methylpyridine (page 197), 4-hydroxy-2-hydroxymethyl-5-methoxypyridine (page 192), 2-(1-n-hexyl)-4-hydroxypyridine (page 187) and 6-(1-n-hexyl)-2-hydroxypyridine (page 188) had very strong absorptions at 1630, 1621, 1628 and 1640 cm^{-1} respectively, typical of 1-(H)-pyridones (page 37), with weaker absorptions in the 3200-3400 cm^{-1} region attributable to

N-H stretching vibrations.

Piericidin A in solution exhibited strong absorptions at 3505 cm^{-1} and 1595 cm^{-1} attributed to the free O-H stretching vibration and a ring vibration respectively of a pyridinol. As already mentioned (page 44) the weak absorption at 1620 cm^{-1} in the IR spectrum of piericidin A is attributed to olefinic C=C stretching vibrations.

Since it appears, as expected (page 30), that the attachment of alkyl and methoxyl groups to the nucleus of 4-hydroxypyridine does not lead to a predominance of the pyridinol tautomer in preference to the 1-(H)-pyridone tautomer in these compounds, and that 3-hydroxypyridines bearing such substituents are typically pyridinols, it is concluded that piericidin A, clearly a pyridinol, is itself a 3-hydroxypyridine.

(v) Comparisons of ultra-violet spectra

The UV spectra of hydroxypyridines and related compounds in acidic, basic and neutral solutions containing 75% methanol (pages 209-211) indicated that the observations made earlier (pages 34-37) concerning the spectra of such compounds in wholly aqueous solutions could be safely extended to this methanolic solvent. It was necessary to use such a solvent in order to make comparisons with the piericidins, which were not sufficiently soluble in water alone to obtain their UV spectra.

6-(1-n-Hexyl)-2-hydroxypyridine had very similar absorptions in acidic, basic and neutral solutions, typical of a 1-(H)-2-pyridone. The alkyl substituent produced a bathochromic shift relative to the spectrum of 2-hydroxypyridine.

Four substituted 3-hydroxypyridines (5-hydroxy-2-methylpyridine, 4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine, 4,6-dimethoxy-2,5-dimethyl-3-hydroxypyridine and 2,4-dimethyl-3-hydroxy-6-methoxypyridine) all displayed bathochromic shifts of their absorption maxima in acidic and basic solutions compared to those in neutral solution. The shifts were accompanied by increases of extinction coefficient, these being most marked in acidic solution. 3-hydroxypyridine itself behaved similarly to the above mentioned compounds, no absorption attributable to the zwitterion (see page 35) being observed. The effect of substituents on the absorption maximum obeyed no simple rule, although all the substituted compounds exhibited a bathochromic shift relative to 3-hydroxypyridine. This unpredictability is well illustrated by the hypsochromic shift in the absorption maximum observed on increasing the substitution of 5-hydroxy-2-methylpyridine to that of 4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine.

4-Hydroxy-2-methylpyridine, 2-(1-n-hexyl)-4-hydroxypyridine and 4-hydroxy-2-hydroxymethyl-5-methoxypyridine all displayed hyprochromic shifts in acidic and basic solution relative to their absorptions in neutral solution, typical of 1-(H)-4-pyridones (page 36).

The absorption maximum at 267 nm in the spectra of the unacetylated piericidins in neutral solution undergoes a bathochromic shift with an increase of extinction coefficient in acidic solution.

This is typical of a 3-hydroxypyridine but not of a 1-(H)-4-pyridone. On the other hand the absence of any absorption maximum above 220 nm in the spectrum of octahydropiericidin A in basic solution is atypical of any known hydroxypyridine.

Thus it is concluded that, although the UV spectra of the piericidins are not completely understood and the possibility that they are 4-pyridinol compounds cannot be excluded, their behaviour is most like that of 3-hydroxypyridines.

(vi) Mass spectral comparisons

During the course of work described in this thesis the mass spectra of a number of pyridine compounds exclusively substituted with alkyl, methoxyl and hydroxyl (or tautomeric hydroxyl) groups were recorded. Additionally the mass spectra of a number of similar commercially available pyridines were recorded. These spectra are presented in the mass spectral appendix in four groups:

(a) Pyridines possessing methyl substituents only (figure 41)

(b) Pyridines possessing an α-(1-n-hexyl)substituent (figure 42)

(c) Pyridines possessing methoxyl (or N-methyl)functions except those which also possess hydroxyl (or 1-(H)-pyridone) functions (figures 43 and 44)

(d) Pyridines possessing hydroxyl (or 1-(H)-pyridone) functions (figure 45)

(a) Pyridines possessing methyl substituents only (figure 41)

The spectra of all these compounds show peaks attributable to the loss of hydrogen and methyl radicals from the molecular ion, $(M)^+$, at m/e = (M-1) and (M-15).

The spectra of all three picolines have peaks at m/e = (M-27), (M-28) and (M-42) corresponding to the elimination of hydrogen cyanide from the ions $(M)^+$, $(M-1)^+$ and $(M-15)^+$. It is also apparent that the loss of a methyl radical from the molecular ion is most favoured in the case of α-picoline, and of a hydrogen radical in the case of β-picoline.

The four lutidines and the collidine examined show an increased tendency to lose a hydrogen radical from the molecular ion, particularly when this has a β-methyl group. The spectra of all five compounds have peaks at m/e = (M-27) and (M-42) as in the case of the picolines and presumably of a similar origin, but those at m/e - (M-27) are of comparatively low intensity. However although the loss of hydrogen cyanide from the molecular ion appears to be less frequent an occurrence, a series of peaks at m/e = (M-41) indicate that the elimination of methyl cyanide from the same is much more common than in the case of the picolines.

In all the spectra, peaks of low intensity due to the doubly charged molecular ion, $(M)^{2+}$, were detected at m/e = $(\frac{M}{2})$.

(b) Pyridines possessing an α-(1-n-hexyl) substituent (figure 42)

All five compounds examined exhibited a base peak at m/e = (M-70), corresponding to the elimination of n-pent-1-ene from their molecular ions *via* a McLafferty rearrangement. This was expected in the cases of 2-(1-n-hexyl)pyridine, 2-(1-n-hexyl)-4-methoxypyridine and 2-(1-n-hexyl)-6-methoxypyridine, but it was supposed (see page 45 and figure 11) that 2-(1-n-hexyl)-4-hydroxypyridine and 6-(1-n-hexyl)-2-hydroxypyridine, being 1-(H)-pyridones,

might behave differently. The fact that they did not may indicate that the molecules exist as pyridinols in the vapour phase. At any rate it can definitely be said that no distinction, based on the absence or presence of ions resulting from a McLafferty rearrangement in the mass spectrometer, can be made between α, β- and γ-hydroxypyridines bearing a long hydrocarbon side-chain in an α-position.

In addition to the base peak mentioned above, and the molecular peak, the spectrum of each compound showed peaks equivalent to the loss of methyl, ethyl, n-propyl and n-butyl radicals from the molecular ion. Ions due to the loss of n-pentyl radicals, at m/e = (M-71), were of negligible intensity compared to those at m/e = (M-70) formed via a McLafferty rearrangement

The breakdown of these ions at m/e = (M-70) appeared typical of the pyridine nucleus bearing the substituents peculiar to each compound, supposing that the residue of the hexyl substituent was present as a methyl function. For instance, that of 2-(1-n-hexyl) pyridine appeared to lose a methyl radical (peak at m/e = 78) or hydrogen cyanide (peak at m/e = 66); those of the two methoxy-pyridines appeared to lose formaldehyde (peak at m/e = 93) or an acetyl radical (peak at m/e = 80); those of the two hydroxypyridines appeared to lose carbon monoxide or a formyl radical (peaks at m/e = 81 and 80).

No peaks corresponding to doubly charged molecular ions, $(M)^{2+}$, or even doubly charged ions of the base peak, $(M-70)^{2+}$, were detected in the spectra of any of these compounds. This was in

contrast to most of the spectra of other pyridine compounds recorded, with the exception of the piericidins which also have a long hydrocarbon side-chain and a molecular peak of correspondingly low intensity.

(c) Pyridines possessing methoxyl (or N-methyl)functions but not hydroxyl (or 1-(H)-pyridone) functions (figures 43 and 44)

The mass spectra of 2-,3-,4-methoxypyridines differ considerably. The molecular ion of 2-methoxypyridine readily eliminates a hydrogen radical, giving a second base peak at m/e = 108, and formaldehyde giving an intense peak at m/e = 79. In contrast the ions at m/e = 108 and 79 in the mass spectrum of 3-methoxypyridine are of low intensity, the principal fragmentation of the molecular ion being the elimination of 43 mass units, corresponding to an acetyl radical (Cf. the methoxyquinolines,[137] page 40), producing a peak at m/e = 66. The ion at m/e = 108 in the mass spectrum of 4-methoxypyridine is of low intensity. The molecular ion appears to decompose chiefly by the elimination of formaldehyde, and less frequently by the elimination of an acetyl radical producing peaks at m/e = 79 and 66 respectively. In all three compounds the elimination of a methyl radical from the molecular ion is infrequent.

The molecular ions of 1-methyl-2-pyridone and 1-methyl-4-pyridone decompose principally by the elimination of carbon monoxide or of a formyl radical.

The peak at m/e = 80 in the mass spectrum of 5-methoxy-2-methylpyridine is attributed to the elimination of an acetyl radical from the molecular ion, similar to the case of 3-methoxypyridine.

Additional peaks at m/e = 108 and 95 correspond to the loss of a methyl radical (presumably from the 2-methyl group) and of carbon monoxide respectively from the molecular ion.

The mass spectra of 2,6-dimethoxypyridine, 4,6-dimethoxy-2,3-dimethylpyridine and 4,6-dimethoxy-2,5-dimethylpyridine all have intense peaks at m/e = (M-1), corresponding to the loss of a hydrogen radical from the molecular ion. This appears to be typical of α-methoxypyridines. Peaks are found in the mass spectra of all three compounds at m/e = (M-30) and (M-45), attributed respectively to the loss of formaldehyde and to the consecutive loss of formaldehyde and a methyl radical from the molecular ions. Peaks of low intensity at m/e = 121 and 124 in the mass spectrum of 2,6-dimethoxypyridine are probably due to the elimination of water and of a methyl radical from the molecular ion of this compound. The mass spectra of the two isomeric dimethoxydimethylpyridines differ radically in one respect. In the case of the 2,5-dimethyl compound the elimination of a methyl radical from the molecular ion producing an intense peak at m/e = 152 is highly favoured. The same process is almost absent in the 2,3-dimethyl isomer, the intensity of the peak at m/e = 152 being very low. Possibly due to the absence of a competitive pathway of decomposition the ion at m/e = 166 in the spectrum of this compound, due to the elimination of a hydrogen radical from the molecular ion, is relatively more intense. The essential difference between these two compounds lies in the location of the β-methyl group. It is tempting to suppose that it is this β-methyl group which is lost from the molecular ion of the 2,5-dimethyl isomer,

although why this should be so much more facile than in the case of the 2,3-dimethyl isomer is not apparent.

The mass spectra of the two remaining compounds, 4-methoxy-2-methoxymethyl-3-methylpyridine and 4-methoxy-2-methoxymethyl-5-methylpyridine, illustrate the ease with which the McLafferty rearrangement takes place. The elimination of formaldehyde by this mechanism from the molecular ion of each accounts for the base peaks at m/e = 137. The elimination of a methyl radical appears to take place from both the molecular ion and the base peak ion of each compound producing peaks at m/e = 152 and 122. The peak at m/e = 107 in the spectrum of the 3-methyl isomer is probably due to the further loss of formaldehyde from the ion at m/e = 137, although why the intensity of the same ion is so much lower in the spectrum of the 5-methyl isomer is not readily explained.

In all the spectra of this group, peaks of low intensity due to doubly charged molecular ions, $(M)^{2+}$, or in the case of the latter two compounds due to doubly charged base peak ions, were detected at m/e = $(\frac{M}{2})$ or 67.5 respectively.

(d) Pyridines possessing hydroxyl (or 1-(H)-pyridone) functions (figure 45)

The mass spectra of many hydroxypyridines have intense molecular peaks, with peaks of relatively low intensity elsewhere, indicating that the molecular ions are particularly stable. The mass spectra of 2-,3- and 4-hydroxypyridine have been reported[131], (page 39). All three compounds eliminate carbon monoxide from their molecular ions, although the elimination of hydrogen cyanide is

of comparable importance in the cases of the 3- and 4-isomers.

This elimination of hydrogen cyanide appears to be peculiar to these two hydroxypyridines, since it is of negligible importance in the cases of 4-hydroxy-2-methylpyridine and 5-hydroxy-2-methylpyridine. The molecular ions of these two compounds appear to eliminate formyl radicals (peaks at m/e = (M-29)) as the major process of decomposition. The elimination of methyl radicals from the molecular ions of both compounds also occurs to a lesser extent.

The mass spectra of the other four α- and β-hydroxy pyridines considered have peaks at m/e = (M-28), (M-30) and (M-29) corresponding to the elimination of carbon monoxide, formaldehyde and formyl radicals from the molecular ions. The elimination of hydrogen cyanide from the same is of relatively little importance since no peaks at m/e = (M-27) were detected.

A peak at m/e = 82 in the mass spectrum of 2-hydroxy-3-methoxypyridine is thought to be due to the loss of an acetyl radical from the molecular ion, in comparison with the case of 3-methoxypyridine (page 116).

Equivalent peaks, at m/e = (M-43), are also observed in the mass spectra of 4,6-dimethoxy-2,3-dimethyl-5-hydroxy pyridine and 4,6-dimethoxy-2,5-dimethyl-3-hydroxypyridine. Both mass spectra are very similar, displaying a large number of common ions with similar intensities. These are listed below, together with an interpretation of their origin.

Peak at m/e =		Species lost from molecular ion
183	(M)	None
182	(M-1)	($H^{\bullet}$)
168	(M-15)	($^{\bullet}CH_3$)
165	(M-18)	(H_2O)
164	(M-19)	($H^{\bullet}$) and (H_2O) consecutively
154	(M-29)	($^{\bullet}CHO$)
153	(M-30)	(CH_2O) or ($H^{\bullet}$) and ($^{\bullet}CHO$) consecutively
152	(M-31)	($H^{\bullet}$) and (CH_2O) consecutively
150	(M-33)	($^{\bullet}CH_3$) and (H_2O) consecutively
140	(M-43)	($^{\bullet}CO.CH_3$)
139	(M-44)	($H^{\bullet}$) and ($^{\bullet}CO.CH_3$) consecutively
		or ($^{\bullet}CH_3$) and ($^{\bullet}CHO$) consecutively
138	(M-45)	($^{\bullet}CH_3$) and (CH_2O) consecutively
137	(M-45)	($H^{\bullet}$), ($^{\bullet}CH_3$) and (CH_2O) consecutively

In the mass spectra of all the hydroxypyridines examined, peaks of low intensity corresponding to doubly charged molecular ions, $(M)^{2+}$ were detected at m/e = $(\frac{M}{2})$.

It is concluded from the above examples that no distinction can be expected between the mass spectra of isomeric pyridines possessing in the α-positions a methoxyl group and a long hydrocarbon side-chain, and in the β- and γ positions a methoxyl group, a methyl group and a hydroxyl group. This means that the location of the β- and γ substituents of the pyridine nucleus of the piericidins cannot be deduced from the mass spectra of the same. As mentioned earlier

(pages 45, 57, 101), mass spectrometry confirms the location of the hydrocarbon side-chain to be in an α-position in the heterocycle, since only with the side-chain in this position are the observed McLafferty rearrangements possible.

(vii) The base-catalysed deuterium exchange of protons in the methyl groups of pyridines

Studies of base-catalysed deuterium exchange of protons in the methyl groups of picolines in alcoholic solution give the sequence of reactivity as γ-picoline>α-picoline>β-picoline[176]. These results have been extended to include the methyl groups of more complex pyridines using aqueous media (pages 213-215). A preliminary experiment performed at 100°C gave no detectable isotopic exchange. It was therefore decided to employ a temperature of 180°C, having first ascertained that the isomerisation of methoxypyridines at this temperature was not excessive (page 212), since some of the compounds to be examined were of this type.

The greater reactivity of protons in the methyl groups of α-picoline and γ-picoline, compared to those of β picoline, was confirmed, isotopic scrambling being essentially complete in the former two cases, but only 18% in the latter. Under the same conditions 5-methoxy-2-methylpyridine exhibited only 67% scrambling in the protons of its α-methyl group. Presumably the decrease in reactivity is caused by the electron donating effect of the methoxyl group. In contrast nearly complete isotopic scrambling was observed in the α-methyl group of 5-hydroxy-2-methylpyridine under the same

conditions. This result is unexpected since the compound was presumably present predominantly as its phenolate anion, from which proton loss would be unlikely. The exchange must have occurred on traces of the neutral molecule present, the apparently greater reactivity in comparison with the corresponding methoxy-compound possibly being on account of the greater homogeneity of the reaction mixture.

Once again under the same conditions, 8% of the protons in the α-methyl group of 4,6-dimethoxy-2,3-dimethylpyridine were found to be isotopically exchanged, the marked reduction in their reactivity being attributed to the presence of electron donating substituents. Increasing the time of reaction and the strength of the base raised this percentage to 75, the exchange of protons in the β-methyl group remaining essentially zero. The additional presence of dioxan was in order to achieve a more homogeneous reaction mixture. The above result (much greater isotopic exchange in an α-methyl group than a β-methyl group in the same compound) is reinforced by the results obtained with 4,6-dimethoxy-2,5-dimethylpyridine, 4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine and 4,6-dimethoxy-2,5-dimethyl-3-hydroxypyridine, although the apparent variations in the degree of exchange of the protons in the α-methyl groups of each, and hence in their reactivities, is not readily explained.

It is concluded that the base-catalysed deuterium exchange of protons in the methyl groups of comparable pyridine compounds is more rapid when these are in an α or a γ-position than when they are in a β position.

In the examples of 4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine and 4,6-dimethoxy-2,5-dimethyl-3-hydroxypyridine, which are isomeric with the pyridine nucleus of the piericidins, virtually no exchange was observed in the protons of the β-methyl groups, whereas considerable exchange took place in those of the α-methyl groups.

An analogous experiment was performed with octahydropiericidin A, which is believed to contain either an γ or a β-methyl group (page 51 and figure 12), in which no isotopic exchange was detected. This was unfortunately a negative result, and although it would be expected were the methyl group in a β position, it cannot be excluded assuming that the methyl group is in the γ position. For instance it is conceivable that a γ-methyl group might be forced out of the plane of the pyridine ring by adjacent substituents, decreasing its reactivity and likelihood of taking part in isotopic exchange. It is also possible that the long hydrocarbon side-chain might affect the reactivity of the medium (Cf. pages 83-84).

It is concluded that the heterocyclic methyl group of the piericidins may be in an γ or a β-position, and that the result of the attempted isotopic exchange reaction on octahydropiericidin A does not exclude either possibility.

(viii) Spray tests

Colour reactions to the Dragendorff reagent[215] were highly varied (page 216). A positive reaction of some sort was usually found with aromatic and aliphatic amines, although three exceptions were found. Piericidin A and other hydroxypyridines all gave colours with the reagent. Amongst the compounds giving no

visible reaction were amides and amino-acids (except for glycine). In both of these cases the basicity of nitrogen is less than in most amines, probably accounting for the lack of reaction. Clearly the reagent does not distinguish different hydroxypyridines and is therefore of no use in the characterisation of piericidin A in this respect.

Both the Folin-Denis reagent[143], and the "enol" spray[216] gave blue colours with all phenols tested, including pyridinols and piericidin A (pages 216 - 217). 1-(H)-pyridones gave no colour reaction or only a very slowly produced one in both cases. As pointed out earlier[141,144] (page 42), a positive reaction to a reagent of this type does not exclude the possibility of the compound being a 2- or 4-hydroxypyridine if such a compound should possess an enolic hydroxyl group (e.g. should it be a 4-pyridinol). It follows that piericidin A behaves as a pyridinol towards both reagents, although the position of substitution of the hydroxyl group in the heterocycle cannot be deduced.

SYNOPSIS CONCERNING THE STRUCTURE OF PIERICIDIN A

It was concluded that there were six possibilities for the correct structure of the pyridine nucleus of piericidin A (page 51 and figure 12, I-VI). Of these, I and II are 4-hydroxypyridines and would be expected to exist as 1-(H)-4-pyridones. Since piericidin A behaves as a pyridinol (page 46), it is unlikely that either I or II is the correct structure. Of the remaining four possibilities, V and VI, where R=H, have been synthesised and do not correspond to piericidin A, the correct structure of which must consequently be represented by III or IV.

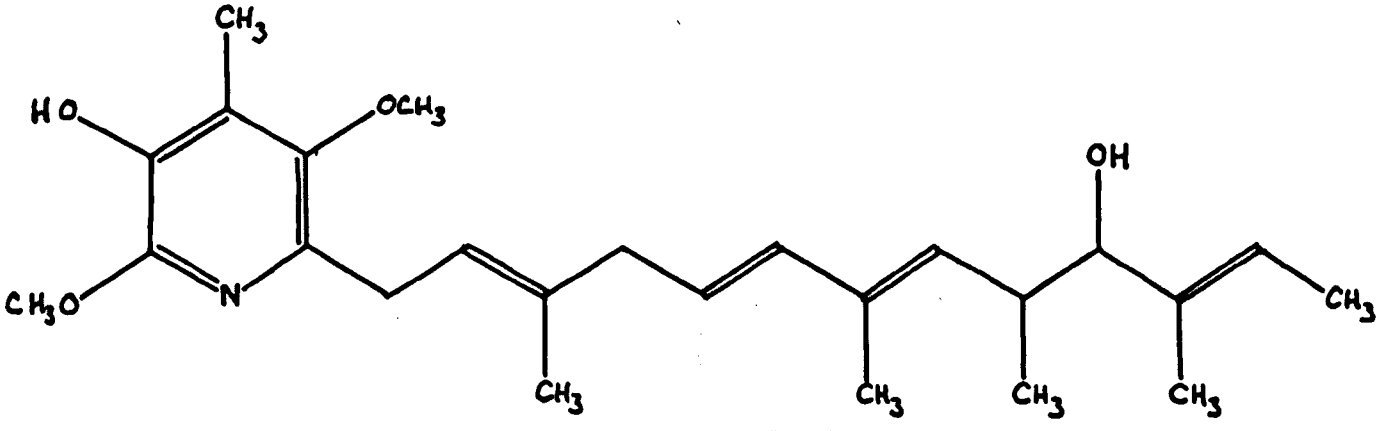
In a parallel investigation[213], the synthesis of 2,4-dimethyl-3-hydroxy-6-methoxypyridine has been achieved. This corresponds to III except that it lacks a methoxyl group. Its UV spectrum has been recorded and does not resemble piericidin A closely (page 210). The ozonolysis of piericidin A diacetate was also examined[213]. A compound identified as the pyridine-2-aldehyde corresponding to piericidin A, but lacking the side-chain, was isolated. In particular it exhibited an aldehydic absorption in its IR spectrum at 1715 cm^{-1}. From this it was concluded that the aldehydic function (and therefore the side-chain) was not adjacent to a phenolic function, since if this

were the case the aldehydic absorption would be expected to be about 50 cm^{-1} less[224]. Both of the above pieces of evidence suggest that the structure III does not correspond to that of piericidin A.

It is therefore concluded, by elimination, that piericidin A is a 2-alkyl-3,6-dimethoxy-5-hydroxy-4-methylpyridine corresponding to structure IV of figure 12.

It is also concluded from the mass spectrum of piericidin A (page 54) that there is an olefinic bond between carbon atoms 5 and 6 of the side-chain, rather than between carbon atoms 4 and 5 as published[2e].

REVISED STRUCTURE OF PIERICIDIN A



EXPERIMENTAL SECTION

Solvents were purified and dried by conventional means.

Commercially available starting materials were either redistilled or recrystallised before use.

Chromatography

Vapour phase chromatography was carried out using a Perkin-Elmer F11 instrument having a flame ionisation detector.

Thin layer chromatography was carried out using the following systems:

	Plate	Eluent (v/v)
System A	Silica	Wet benzene/acetone, 25:1
B	Silica	chloroform/acetone, 7:3
C	Silica	chloroform/methanol/water, 65:25:4
D	Silica	dry benzene
E	Silica	dry benzene/acetone, 19:1
F	Silica	ethyl acetate
G	alumina	dry benzene
H	alumina	dry benzene/acetone, 19:1

The detection of materials on developed T.L.C. plates was by exposure to iodine vapour unless otherwise stated.

Spectrometers

Ultra-violet spectra were recorded using a Unicam SP 800 instrument. Extinction coefficients at the maximum of absorbance were checked using either a Unicam SP 500 or a Carey 14 instrument.

Infra-red spectra were recorded using a Perkin-Elmer 257 instrument.

^{1}H NMR spectra were recorded at 60 Mc/s and 14,100 gauss using a Perkin-Elmer R10 instrument.

Mass spectra were recorded using an A.E.I. M.S. 902 mass spectrometer at the University of Hull, on a service basis. Direct insertion of the samples was employed.

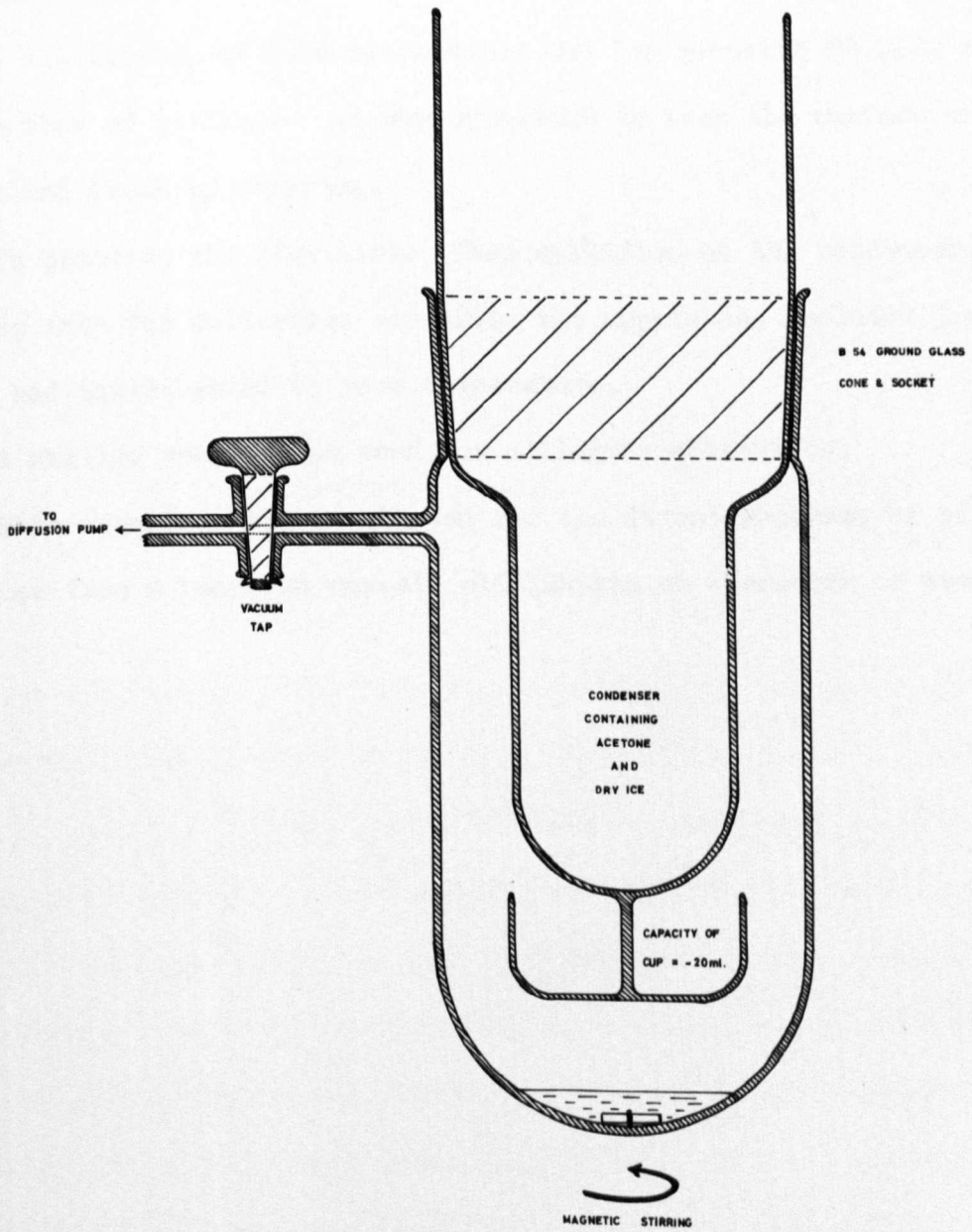
Melting Points are given uncorrected.

The radioactivity of samples containing tritium was measured using a Packard Tri-Carb liquid scintillation spectrometer.

Elemental Analyses were determined commercially.

FIGURE 34

APPARATUS FOR SHORT PATH DISTILLATION



Short path distillation

The glass apparatus indicated in figure 34 was developed for the purification of liquids having an appreciable vapour pressure at room temperature.

The best results were achieved by a process of slow evaporation of the distilland, at room temperature and low pressure (c.0.01 mm Hg), rather than of boiling. It was important to keep the surface of the distilland fresh by stirring.

In practice the distillate often solidified on the condenser, only running into the collection cup after the apparatus, isolated from the pump, had equilibrated to room temperature.

A smaller version was used for milligram quantities.

This apparatus may be adapted for the direct recovery of oily residues from a reaction vessel, eliminating an operation of transfer.

METABOLITES OF STREPTOMYCES MOBARAENSIS AND THEIR DERIVATIVES

Streptomyces mobaraensis[1] was grown on two different culture media.

Rich medium[1]

Composition:	glucose	20 g/l
	starch	10 "
	soya peptone	25 "
	meat extracts	10 "
	yeast extract	4 "
	sodium chloride	2 "
	dipotassium hydrogen phosphate	0.5"
	10% sodium hydroxide solution	3 ml

The conditions of the fermentation and of the extraction of piericidins A and B were substantially the same as those of Takahashi et al[1]. After a typical 5l. fermentation, silicic acid (Mallinckrodt) column chromatography yielded piericidin B (18 mg) in the eluent containing 1½% ethyl acetate in benzene, and piericidin A (65 mg) in the eluent containing 3% ethyl acetate in benzene. Piericidins A and B so isolated were ascertained by T.L.C. (System A) to be single compounds.

Apart from fatty substances, eluted early from the column, no other metabolites were isolated from mycelia of S.mobaraensis grown on this medium.

Simple medium[156]

Composition:		
	glucose	20 g/l
	bacteriological peptone	5 "
	sodium chloride	2 "
	dipotassium hydrogen phosphate	2 "

Other conditions of the fermentation and of the extraction of metabolites were the same as those mentioned above. Piericidin B (18 mg) and piericidin A (85 mg) were isolated as above from a typical 5 l. fermentation. In addition two new non-fatty metabolites were isolated in eluents from the silicic acid column. Metabolite C (20 mg) was found in that containing 2% ethylacetate in benzene (that is, between piericidins A and B), and metabolite D (10 mg) was eluted earlier with benzene. Both were contaminated with other materials and were purified by preparative T.L.C. (System A) yielding 9 mg and 5 mg respectively.

Piericidin A

A viscous pale yellow oil

Chromatographic homogeneity

TLC System A	One spot, R_f = 0.22 sensitive to "enol" spray

UV Spectra	λ max (nm)	ϵ max
(75% methanol, 25% water)	232	39,500
	239	40,500
	267	5,600
(75% methanol, 25% 0.1NHCl)	236	39,000
	273	8,200

FIGURE 35

^{1}H NMR SPECTRA

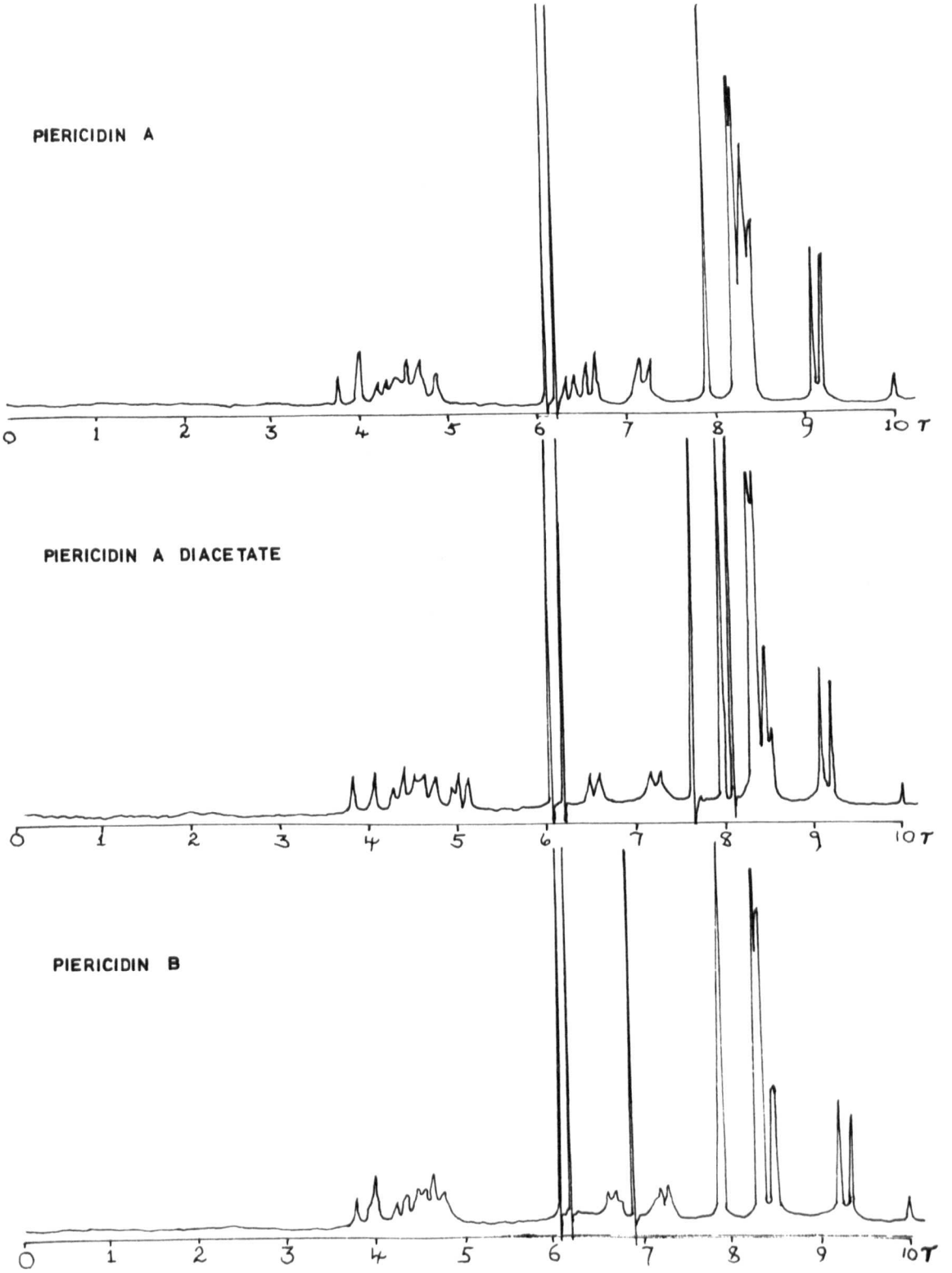
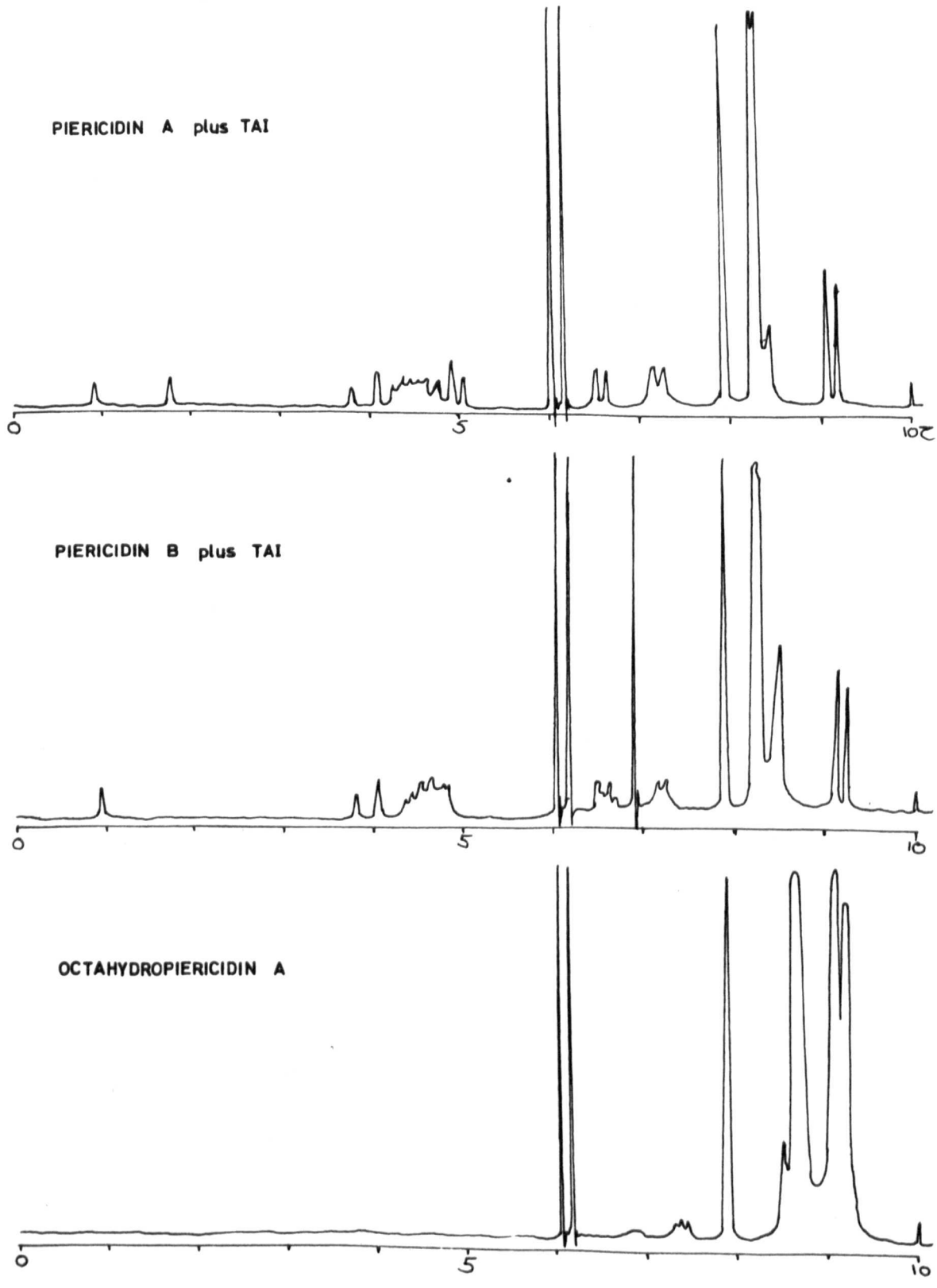


FIGURE 36

^{1}H NMR SPECTRA



(75% methanol, 25% 0.1N NaOH) 237 40,000

Shoulder 3,200

at 270 nm.

IR Spectra

ν ($CHCl_3$) cm^{-1} 3505 (st. and sharp), 2925, 2870 (med)

1620 (w), 1595, 1475, 1420 (st.)

1390, 1360, 1330,(w), 1240, 1190 (med.)

1128 (st.), 1042, 1005, 968 (med.)

ν (thin film) cm^{-1} 3380 (broad st.), 2025, 2865 (med.), 1620 (w), 1595

1475, 1420 (st.), 1390, 1360, 1330 (w)

1252, 1193 (med.), 1129 (st.), 1050, 1008.

968. 040, 914, 882, 829, 760 (all med.)

^{1}HNMR Spectra

τ ($CDCl_3$) See figure 35

3.87 (d,J=15.5 H_z)1H,

4.19-4.29-4.45-4.56-4.60-4.86 (m.)4H,

6.08 (s) 3H, 6.18 (s)3H, 6.36 (d,J=8.9 H_z)1H,

6.63 (d,J=6.9 H_z)2H, 7.20 (d, J=6.5 $H_z$and m)

3H, 7.91 (s)3H, 8.15-8.45 (m)12H,

9.19 (d, J=6.8 H_z)3H

τ ($CDCl_3$ plus TAI) See figure 36

0.93 (s)1H, 1.77 (s)1H, 3.94(d,J=15.5H_z)1H,

4.26-4.35-4.47-4.63-4.75-4.90 (obscured),

(m)4H, 4.99(d,J=8.9 H_z)1H, 6.04 (s)3H,

6.18 (s)3H, 6.60 (d,J=6.9 H_z)2H

7.25 (m.and d., J= 6.5 H_z)3H,

7.94 (s)3H, 8.20-8.45 (m)12H,

9.14 (d,J=6.8 H_z)3H

Mass Spectrum

See figure 40 of the mass spectral appendix

No peaks due to metastable transitions detected

Accurate mass measurements:

331.213 ($C_{20}H_{29}NO_3$ is 331.2147)

236.130 ($C_{13}H_{18}NO_3$ is 236.1286)

222.113 ($C_{12}H_{16}NO_3$ is 222.1130)

183.089 ($C_9H_{13}NO_3$ is 183.0895)

161.133 ($C_{12}H_{17}$ is 161.1330)

84.057 (C_5H_8O is 85.0575)

Piericidin B

A viscous pale yellow oil

Chromatographic homogeneity

T.L.C. System A — One spot, R_f=0.33, sensitive to enol spray

U.V. Spectra

	λ max (nm.)	ϵ max
(75% methanol, 25% water)	232	39,500
	239	40,500
	267	5,600
(75% methanol, 25% 0.1NHCl)	236	39,000
	273	8,200
(75% methanol, 25% 0.1N NaOH)	237	40,000
	Shoulder at 270 nm.	3,200

IR Spectrum

ν(thin film) cm^{-1} 3370 (broad st.), 2925, 2870 (med.)
1620 (w), 1595, 1475, 1420 (st.)
1390, 1360, 1330(w), 1250, 1195 (med.)
1128 (st.) 1100 (Med.) 1050, 957, 830
770 (med.)

^{1}HNMR Spectra ($CDCl_3$) See figure 35
3.90 (d, J=15.5 Hz)1H, 4.29-4.40-4.54-4.66-
4.79 (m)4H, 6.08 (s)3H, 6.18 (s)3H,
6.64 (d, J=6.9 Hz) and 6.7 (d, obscured)3H,
6.90 (s)3H, 7.23 (m. and d, J=6.5 Hz)3H,
7.29 (s)3H, 8.20-8.50 (m)12H, 9.22 (d, J=6.8 Hz)3H

τ($CDCl_3$ plus TAI) See figure 36
0.97 (s)1H, 3.93 (d, J=15.5 Hz)1H,
4.31-4.43-4.54-4.64-4.75-4.82 (m)4H
6.03 (s)3H, 6.17 (s)3H, 6.59 (d, J=6.9 Hz)3H,
6.89 (s)3H, 7.22 (m. and d.)3H, 7.92 (s)3H,
8.20-8.45 (m)12H, 9.20 (d, J=6.8 Hz)3H

Metabolite C

White crystalline M.Pt. 199-200°C

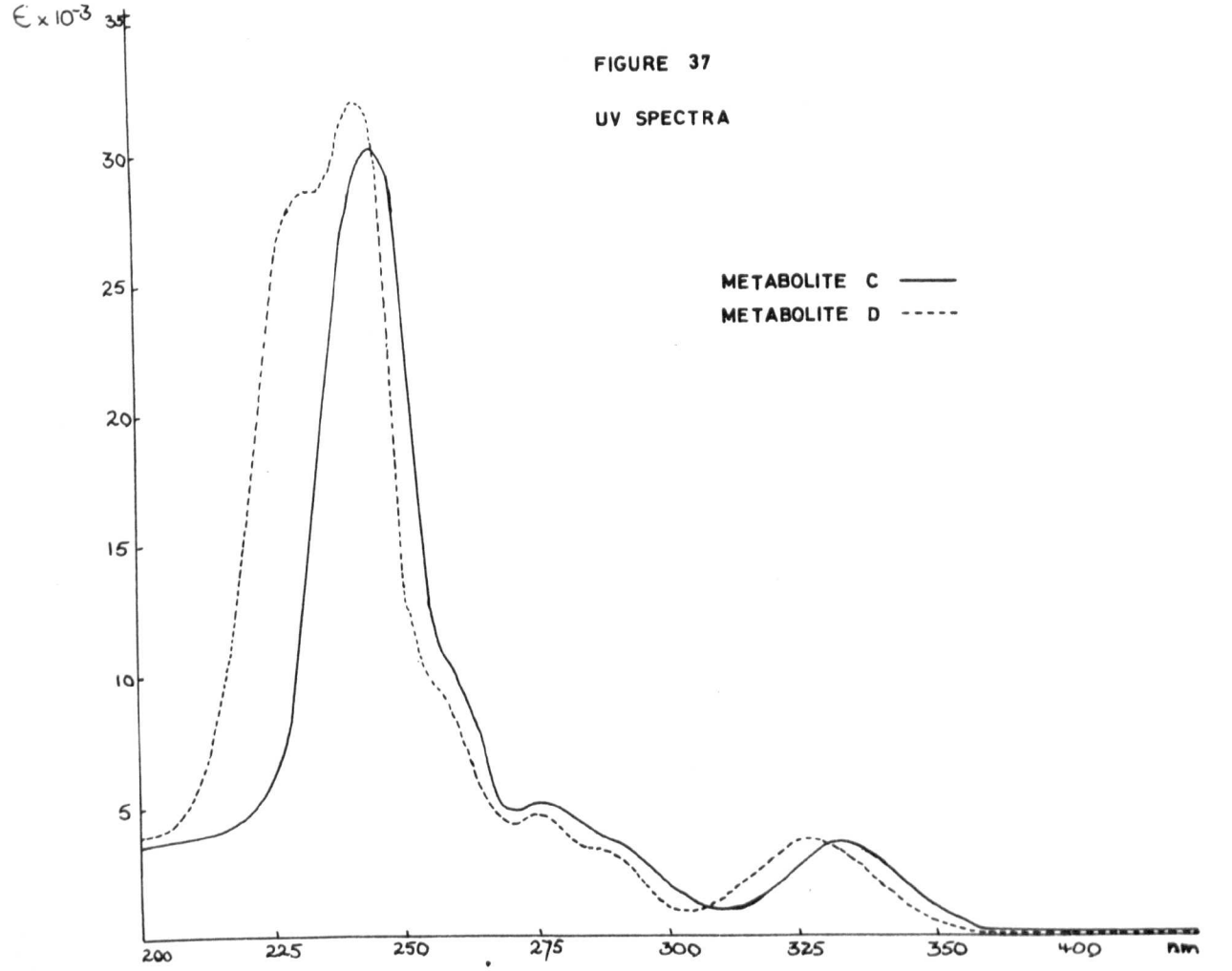
Soluble in water

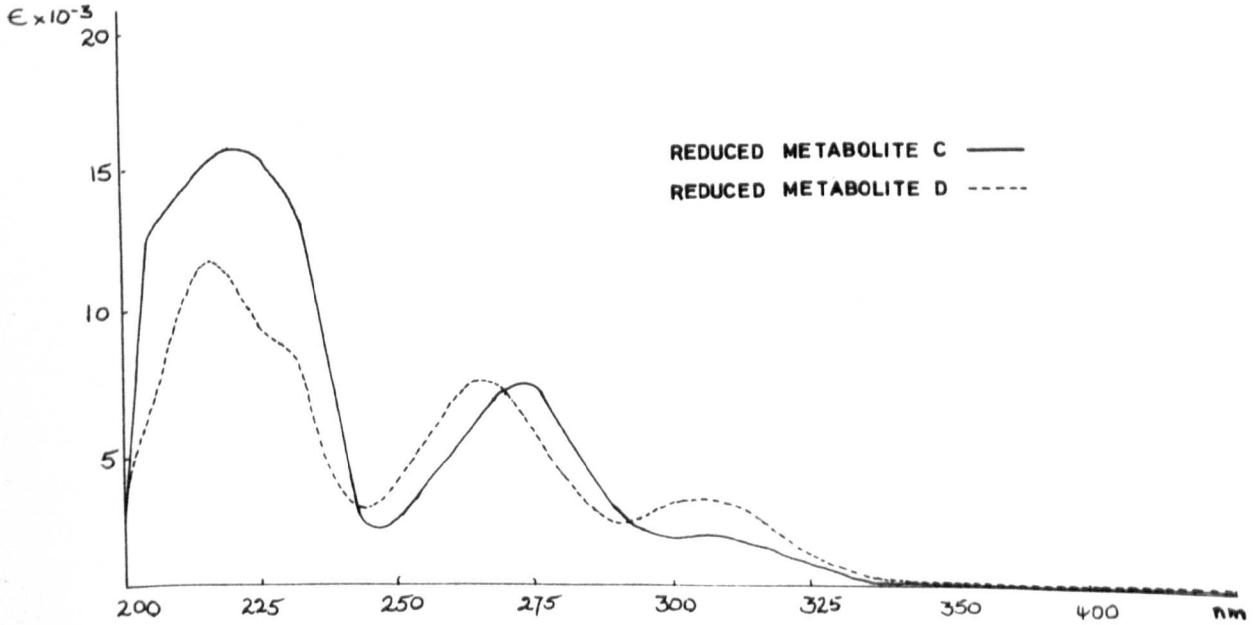
Chromatographic Homogeneity

T.L.C. System A One spot R_f = 0.27 (sensitive to enol spray)

FIGURE 37

UV SPECTRA





UV Spectra

	λ max (nm)	ϵ max	
(methanol)			
(and acidified methanol)	See figure 37		
	242	30,500	based on M.W. of 236
	279	5,100	
	334	3,700	
(methanol plus 2 drops IN NaOH)	234	30,000	
	356	5,000	

IR Spectra

ν ($CHCl_3$) cm^{-1} 2920, 2850 (med.), 1682, 1646(v.st.) 1562, 1460, 1376, 1340, 1290, 1112, 1096, 1002, 979 (all st.)

ν (nujol) cm^{-1} 3400 (broad w.), 1680, 1644, 1561, 1520, 1433, 1355, 1290, 1262, 1210, 1167, 1108, 1020, 1005, 976, 849, 792, 746 (all st.)

^{1}H NMR Spectrum

τ ($CDCl_3$, 2%) -1.12 (s)1H, exchanged by addition of D_2O

3.67 (s)1H, 3.82 (s)1H, 6.05 (s) 3H, 6.09 (s)3H, 7.74 (s)3H, 8.45 (s) 2H, exchanged by addition of D_2O

Mass Spectrum

m/e	I		m/e	I
236	(97)	and after shaking	237	(100)
221	(100)	with D_2O →	222	(97)
207	(17)		208	(13)
193	(76)		194	(73)
150	(19)		151	(16)

Peak due to metastable transition

$221^{+} \longrightarrow 193^{+} + 28$ @ 168.55

Accurate mass determinations

236.0672 ($C_{10}H_{10}N_3O_4$ = 236.0671)

221.0436 ($C_9H_7N_3O_4$ = 221.0436)

193.0499 ($C_8H_7N_3O_3$ = 193.0487)

Metabolite D

White crystalline, M.Pt. 123-125°C

soluble in water

Chromatographic Homogeneity

T.L.C. System A — One spot, R_f = 0.56 (sensitive to enol spray)

UV Spectrum(methanol) — See figure 37

λ max (nm)	ϵ max	
238 (shoulder)	28,500	based on M.W. of 206
244	32,500	
277	4,700	
287	3,200	
326	3,700	

ν ($CHCl_3$) cm^{-1} — 3400 (broad w.), 2920, 2850 (med.)
1685 (v.st.) 1648, 1623, 1573 (all st.),
1490, 1465 (med.) 1372, 1351, 1334 (st.),
1163, 1149 (v. st.), 1099, 1069 (med.),
1004, 971, 950, 888, 844 (all med.)

^{1}H NMR Spectrum

τ ($CDCl_3$, 1%) — -1.12 (s)1H exchanged by addition of D_2O,
3.51 (d,J=2.6 H_z)1H, 3.67 (d,J=2.6 H_z)1H,

3.80 (s)1H, 6.15 (s)3H, 7.76 (s)3H

8.25 (s)1H

Mass Spectrum m/e (I)

206 (100) and after 207 (100)

191 (35) shaking 192 (36)

177 (12) with 178 (14)

135 (32) D_2O ⟶ 136 (36)

Peaks due to metastable transitions

$206^+ \longrightarrow 177^+ + 20$ @ 152.08

$207^+ \longrightarrow 178^+ + 29$ @ 153.06

Accurate mass determination

206.0561 ($C_9H_8N_3O_3$ = 206.0566)

Piericidin A diacetate was prepared in 55% yield by the action of acetic anhydride in pyridine on piericidin A as described by Takahashi et al[2a].

A viscous pale yellow oil

Chromatographic homogeneity

T.L.C. System A — One spot, R_f = 0.74 sensitive to enol spray

UV Spectra	λ max nm	ε Max
(75% methanol, 25% water)	278	6,800
(75% methanol, 25% 0·1NHCl)	280	6,400
(75% methanol, 25% 0.1N NaOH)	270 (shoulder)	3,000

^{1}H NMR Spectrum — See figure 35

τ ($CDCl_3$)

3.94 (d,J=15.5 H_z)1H, 4.27-4.37-4.49-4.62-4.76-4.95 (m)4H, 5.06(d,J=8.9H_z)1H

6.05 (s)3H, 6.20 (s)3H, 6.61 (d,J=6.9H_z)2H
7.24 (m and d, J=6.5 H_z)3H, 7.67 (s)3H,
7.99 (s)3H, 8.08 (s)3H, 8.30–8.50 (m)12H,
9.15 (d,J=6.8 H_z)3H

Octahydropiericidin A was prepared in 90% yield by the hydrogenation of piericidin A in methanolic solution, using a platinum catalyst, as described by Takahashi et al[2a].

A viscous colourless oil

Chromatographic homogeneity

T.L.C. System A — One spot R_f = 0.26 sensitive to enol spray

UV Spectra	λ max (nm)	ϵ max
(75% methanol, 25% water)	267	5,600
(75% methanol, 25% 0.1N HCl)	233	5,600
	274	8,200
(75% methanol, 25% 0.1N NaOH)	240 (shoulder)	9,000
	and 270	2,700

IR Spectrum

ν (thin film) cm^{-1} 3360 (broad st.), 2925, 2870 (med.) 1595, 1475, 1420 (st.), 1390, 1365 1330 (w), 1250, 1186 (med.), 1129 (st.), 1048, 976 (med.), 868, 829, 758 (all med.)

^{1}H NMR Spectrum

τ ($CDCl_3$) 6.07 (s)3H, 6.17 (s)3H, 6.85 (m)1H, 7.40 (t ,J=7.5 H_z)2H, 7.91 (s)3H, 8.55–8.80 (m)16H, 9.00–9.20 (m)15H

Mass spectrum — See figure 40 of the mass spectral

appendix.

Peak due to metastable transition

$183^{+} \longrightarrow 168^{+} + 15$ @ 154.23

Deuterium

Zinc containing 10% tin (3_g) was added to deuterium oxide (10g, 99.7%) acidified with redistilled sulphuryl chloride (2g) contained in an evacuated vessel at room temperature. Deuterium gas (1 litre at S.T.P.) was slowly evolved and collected over natural water.

Mass spectrometric analysis

m/e	Relative Intensity [Corrected for background]	Percentage
2	10	0.47
3	190	8.96
4	1920	90.57

Isotopic abundance of deuterium $= \frac{(D)}{(D)+(H)} \times 100$

$$= \frac{1920 + \frac{190}{2}}{2120} \times 100$$

$$= 95.06\%$$

Perdeuteropiericidin A was prepared in 90% yield by the hydrogenation of piericidin A in d_1-methanolic solution with deuterium gas, using a platinum oxide catalyst. The method was analogous to that described by Takahashi et al for octahydropiericidin A [2a].

Chromatographic homogeneity

T.L.C. System A — One spot, $R_f = 0.26$

UV Spectrum (methanol)

λ max (nm)	ϵ max
267	5,400

^{1}H NMR Spectrum

τ ($CDCl_3$) 6.07 (s)3H, 6.17 (s)3H, 6.85 (m)1H
7.41 (d,J 7.5 H_z)2H, 7.91 (s)3H,
8.55-8.80 (m)8H, 9.00-9.20 (m)15H

Mass spectra

(1) From ($^1H_4$)-methanolic solution: see figure 40 of M.S.A. for spectrum.

(MSA = Mass spectral appendix)

(2) From ($^2H_1$)-methanolic solution: see figure 40 of M.S.A., prominent differences from the preceeding spectrum are recorded.

Peaks due to metastable transitions:

(1) $184^+ \longrightarrow 169^+ + 15$ @ 155.22

(2) $185^+ \longrightarrow 170^+ + 15$ @ 156.22

Reduction of metabolite C

Platinum oxide (2 mg) was added to metabolite C (10 mg) in methanol (25 ml), and the mixture hydrogenated for 24 hours at one atmosphere pressure and 20°C. Crude hydro-metabolite C (9.2 mg) was recovered after the removal of catalyst and solvent.

Chromatographic homogeneity

T.L.C. System A R_f = 0.19 strong
R_f = 0.27 faint-starting material (both sensitive to enol spray)

Pure hydro-metabolite C (8.5 mg) was recovered after preparative T.L.C.

White, crystalline, M.Pt. 137-139°C

UV Spectra

See figure 37

	λ_{max} (nm)	ϵ_{max}	
(methanol)	221	15,800	based on M.W. of 238
	273	7,500	
	305 (shoulder)	1,800	
(methanol + 2 drops HCl conc.)	221	15,400	
	275	9,500	
	305 (shoulder)	2,200	
(methanol + 2 drops 1N.NaOH)	270 (shoulder)	7,500	
	340	4,000	

IR Spectrum

$\nu(CHCl_3)$ cm^{-1} 2920, 2850 (med.), 1668 (v.st.)

1619, 1579, 1453, 1355, 1265 (all st.)

1120 (v.st.), 975, 940 (med.)

^1H NMR Spectrum

$\tau(CDCl_3, 1\%)$ -1.14 (s)1H exchanged by addition of D_2O

3.64 (d,J=4.3 H_z)1H, 6.10 (s)3H, 6.12(s)3H,

7.13 (d,J=7.0 H_z)2H, 8.51 (d,J=7.0 H_z)2H

9.1 (d,J=7.0 H_z)3H

	m/e (I)		m/e (I)
Mass spectrum	238 (73)	and after	239 (100)
	223 (27)	shaking	224 (19)
	219 (9)	with	220 (14)
	209 (23)	$D_2O \rightarrow$	210 (19)
	205 (37)		205 (51)
	195 (50)		196 (19)
	191 (28)		192 (11)
	179 (47)		180 (26)
	177 (100)		177 (72)
	163 (30)		164 (43)
	151 (37)		152 (16)

Accurate mass determinations:

238.0828 ($C_{10}H_{12}N_3O_4$ = 238.0827)

177.0546 ($C_8H_7N_3O_2$ = 177.0538)

Reduction of metabolite D

Platinum oxide (0.5 mg) was added to metabolite D (2 mg) in methanol (5 ml), and the mixture hydrogenated for 24 hours at one atmosphere pressure and 20°C. Crude hydro-metabolite D (1.8 mg) was recovered after the removal of catalyst and solvent. Examination of this material by TLC (System A) revealed four products (R_f values = 0.10, 0.46, 0.70, 0.75). Hydro-metabolite D (0.5 mg) was recovered from the band at R_f = 0.70 (sensitive to enol spray) after preparative T.L.C.

Waxy solid (amorphous)

UV Spectrum	λ max (nm)	Relative Intensities	ϵ max	
	217	1.00	8–16,000	based on molecular weight of 208
(methanol)	See figure 37			
	267	0.63	5–10,000	
	301	0.26	2–4,000	

IR Spectrum

ν ($CHCl_3$) cm^{-1} 2920, 2850, 1664, 1636 (all st.)

1580 (med.), 1365, 1154

Mass spectrum: m/e (I) 208 (55)

190 (15)

179 (12)

164 (100)

(^{3}H)-Piericidin A

Platinum oxide (700 µg) was added to piericidin A (10 mg) in cyclohexane (0.5 ml) and the mixture stirred under tritium gas (1 Curie) for 24 hours at 20°C in a tritium vacuum line. (^{3}H)-Piericidin A (8.5 mg) was recovered by preparative T.L.C. (System A), R_f = 0.22.

UV Spectrum (cyclohexane)

λ max (nm)	ϵ max
232	39,100
239	40,000

Specific activity 56.5 mC/mmole (corrected for background radiation and self quenching effect).

(^{3}H)-Octahydropiericidin A

Platinum oxide (4 mg) was added to (^{3}H)-piericidin A (4 mg) in

methanol (2 ml), and the mixture hydrogenated at one atmosphere pressure for 24 hours at 20°C. (^{3}H)-Octahydropiericidin A (3.6 mg) was recovered by preparative T.L.C. (System A), R_f = 0.26

UV Spectrum

(methanol)	λ max (nm)	ϵ max
	267	5,400

Specific activity 56.5 mC/mmole (corrected for background radiation and self quenching effect).

Scintillating fluid for use in the Packard Tri-Carb liquid scintillation spectrometer

2,5-diphenyloxazole (PPO, 8g) and 1,4-bis[2-(5-phenyloxazoyl)] benzene (POPOP, 0.5g) were dissolved in toluene (700 ml) and methanol (300 ml). The solution was stored in the dark.

The quenching effects of piericidin A and of octahydropiericidin A on the scintillation counts observed in the liquid scintillation spectrometer

Aliquots of a methanolic solution of (^{3}H)-piericidin A (70 µg/ml) were added to a series of Packard Tri-Carb vials, each containing scintillating fluid (10 ml). The actual counts of each vial were measured after the vials had been left in the dark for a few hours in order to reach equilibrium.

The experiment was repeated using a methanolic solution of (^{3}H)-octahydropiericidin A (100 µg/ml).

The results are shown below, and the quenching curves in figure 38.

A: (^{3}H)-piericidin A (70µg/ml of methanol)

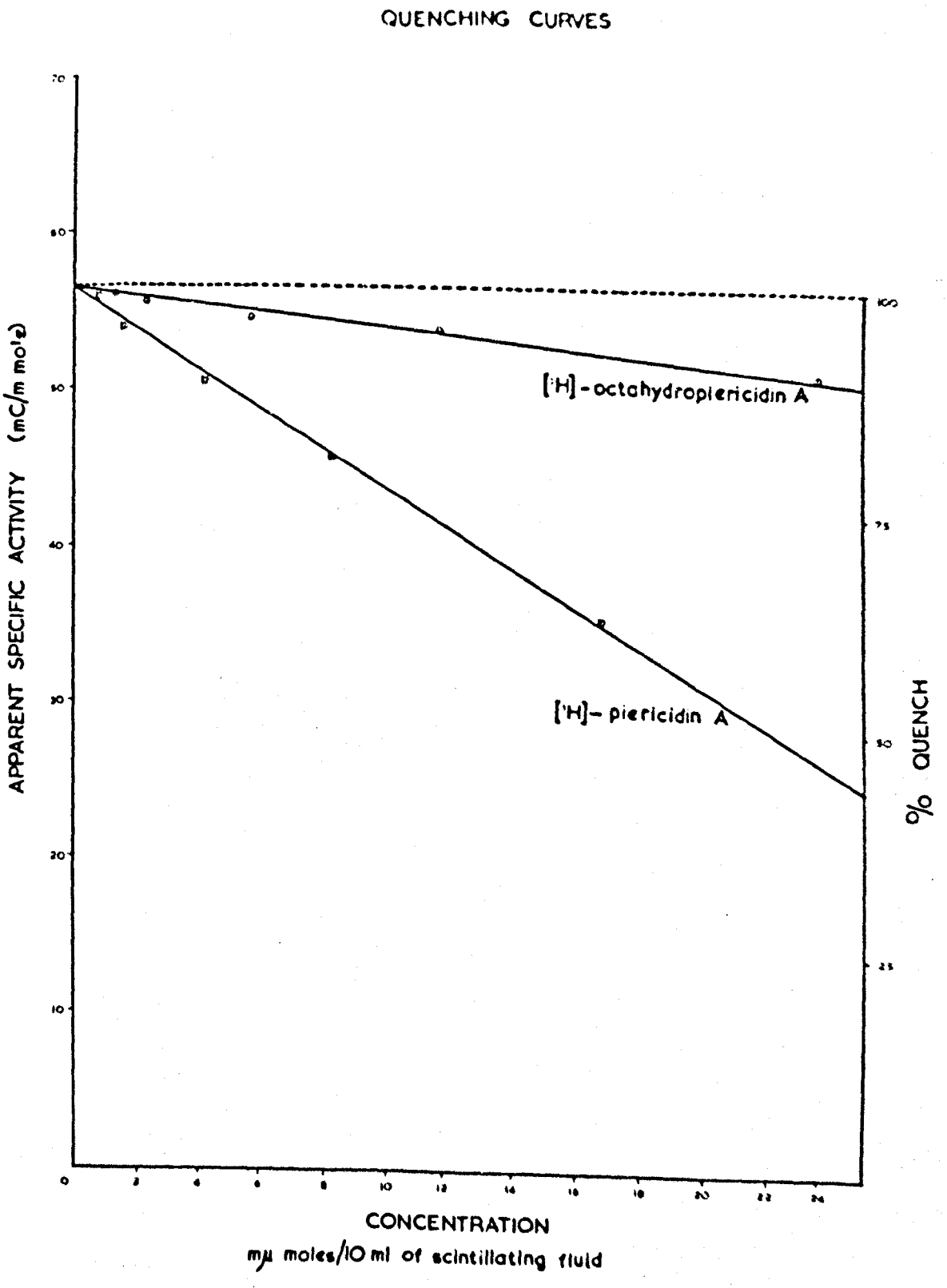
µl of solution added	(^{3}H)-piericidin A added µg	mµmoles	apparent radioactivity cpm	µC	apparent specific activity mC/mmole
0	0	0	100	-	-
5	0.35	0.835	103,300	0.046	55.9
10	0.70	1.685	201,700	0.091	53.9
25	1.75	4.22	472,200	0.213	50.4
50	3.5	8.35	845,100	0.381	45.7
100	7.0	16.85	1,320,000	0.595	35.3

B: (^{3}H)-octahydropiericidin A (100 µg/ml of methanol)

µl of solution added	(^{3}H)-octahydro-piericidin A added µg	mµmoles	apparent radioactivity cpm	µC	apparent specific activity mC/mmole
0	0	0	100	-	-
5	0.5	1.18	147,000	0.066	56.1
10	1.0	2.36	290,600	0.131	55.5
25	5.0	11.8	1,410,000	0.635	53.8
100	10.0	23.6	2,659,000	1.198	50.7

Beef Heart Mitochondria were prepared by the method of Sanadi and Fluarty[217].

FIGURE 38


QUENCHING CURVES
APPARENT SPECIFIC ACTIVITY (mC/m mole)
% QUENCH
[³H]-octahydropiericidin A
[³H]- piericidin A
CONCENTRATION
mμ moles/10 ml of scintillating fluid

EXPERIMENTS OF BIOCHEMICAL SIGNIFICANCE

The inhibition of NADH-linked oxidation

Beef heart mitochondria (0.1 ml, 2-3 mg of protein) were suspended in 0.25M sucrose and 50 mM tris HCl., pH 7.6, (2.8 ml) at 30°C in the cell of an oxygen electrode. After 2-3 minutes, when a steady rate of oxygen uptake was established, 0.1M sodium pyruvate and 0.1M sodium malate, pH 7.6, (0.1 ml) was added, causing a marked increase in respiration as measured by the rate of oxygen uptake.

Inhibitors were added in ethanolic solution, the degree of inhibition being measured by the change in the rate of oxygen uptake, taking into account the slight inhibition caused by ethanol alone.

The results are shown below.

Compound	Concentration required for 50% inhibition μ moles/mg. of protein
piericidin A	1.1×10^{-5}
piericidin A diacetate	1.2×10^{-2}
octahydropiericidin A	2.5×10^{-5}
piericidin B	2.5×10^{-5}
	mM
4-hydroxypyridine	5.5
4-hydroxy-2-methylpyridine	5.0
4-hydroxy-2-hydroxymethyl-5-methoxypyridine	12.5
2,6-dimethyl-4-hydroxypyridine-1-oxide	6.0
vanillin	3.0
4-(1-n-hexyl)-2-methoxyphenol	0.045

4-(1-n-pentyl)phenol	0.11
2-(1-n-hexyl)pyridine	0.35
2-(1-n-hexyl)-4-hydroxypyridine	0.10
6-(1-n-hexyl)-2-hydroxypyridine	0.12
2-(1-n-hexyl)-4-methoxypyridine	1.2
2-(1-n-hexyl)-6-methoxypyridine	0.9
4-chloro-2-(1-n-hexyl)pyridine	0.6
6-chloro-2-(1-n-hexyl)pyridine	0.8

The binding of (^{3}H)- piericidin A to mitochondria

(performed by Mr. A. J. Sweetman)

A. The binding of (^{3}H)- piericidin A to mitochondria in the absence of bovine serum albumen, and the corresponding inhibition of NADH - linked oxidation

Beef heart mitochondria (1-0 ml, 20 mg. of protein) were suspended in 0.25M sucrose and 50 mM tris HCl, pH7.6, (25ml) at 30°C. (^{3}H)-piericidin A was added in ethanolic solution. After 8 minutes the mitochondria were collected by centrifugation at 30,000xg, washed with the sucrose-tris medium, and resuspended in more of the same (4ml). Aliquots of this suspension (x 0.1ml) were added to scintillating fluid (10ml), and the radioactivity measured in a Packard Tri-Carb liquid scintillation spectrometer.

The inhibition of NADH-linked oxidation by (^{3}H)- piericidin A, added at corresponding concentrations, was measured as described in the experiment immediately above.

The results of both the binding and the inhibition experiments are shown in table 4.

TABLE 4

mu moles of (^{3}H)-piericidin A added	A/g of protein bound	Inhibition %
10.4	8.3	42.5
26	22	68.5
52	40	88
104	83	96
156	121	100
208	160	100
1040	680	100

B. The binding of (^{3}H)- piericidin A to mitochondria in the presence of bovine serum albumen, and the effect of repeated washing with bovine serum albumen.

The procedure of the experiment described immediately above was repeated using 0.25M sucrose and 50mM tris HCl,pH 7.6, containing 2% bovine serum albumen in place of the sucrose-tris medium.

The effect of repeated washing with the sucrose-tris-bovine serum albumen medium, by the process of suspension and recollection of the mitochondrial pellets, was also investigated.

The results are shown in table 5.

TABLE 5

mμ moles Added	(^{3}H) piericidin A/g of protein bound after number of washes shown: 0	1	3	5	7	% binding (after 7 washes)	% inhibition (from table 4)
10.4	8.4	8.2	7.4	6.9	6.8	35	42.5
26	18.2	17.7	15.8	14.8	13.4	71	68.5
52	34.3	27.8	22.8	21.2	19.2	100	88
104	51.8	31.4	24.1	20.8	1911	100	96
260	98	-	-	-	-	-	-
520	170	-	-	-	-	-	-
1040	350	-	-	-	-	-	-

C. The binding of (^{3}H)-piericidin A to mitochondria pretreated with unlabelled piericidin A in the presence of bovine serum albumen

Beef heart mitochondria (1.0ml, 20mg. of protein) were suspended in 0.25M sucrose and 50mM tris CCl, pH 7.6, containing 2% bovine serum albumen (25ml) at 30°C, and incubated for 8 minutes with unlabelled piericidin A (0.7 mμ moles). (^{3}H)-Piericidin A was added in ethanolic solution and the suspension incubated for a further 8 minutes. The

mitochondria were collected by centrifugation at 30,000 xg, washed with sucrose-tris-bovine serum albumen medium, and resuspended in more of the same (4ml). Aliquots of this suspension (x0.1ml) were added to scintillating fluid (10ml), and the radioactivity measured in a Packard Tri-Carb liquid scintillation spectrometer.

The results are shown in table 6.

TABLE 6

mμ moles of (^{3}H)-piericidin A added	m/g of protetin bound
10.4	3.9
26	8.0
52	16.4
104	34
260	80
520	165

D. The effect of repeated washing with bovine serum albumen on specifically and unspecifically bound (^{3}H)-piericidin A

(i) Beef heart mitochondria (1.0ml, 20mg of protein) were suspended in 0.25M sucrose and 50mM tris HCl, pH 7.6, containing 2% bovine serum albumen (25ml) at 30°C, and incubated for 8 minutes with unlabelled piericidin A (0.15 mμmoles). (^{3}H)-Piericidin A was added in ethanolic solution and the suspension incubated for a further 8 minutes. The mitochondria were collected and their radioactivity was measured as described in the experiment immediately above. Once again the effect of repeated washing with the sucrose-tris-bovine serum albumen medium was investigated by the process of suspension and recollection of the mitochondrial pellets.

The results are shown in table 7.

TABLE 7

mμ moles added	(^{3}H)-piericidin A/g of protein bound after number of washes down 0	1	2	3
1.3	0.39	0.16	0.10	0.08
2.6	0.83	0.31	0.14	0.10

(ii) The experiment described immediately above was repeated using the same quantities of materials, but reversing the additions of unlabelled piericidin A and (^{3}H)-piericidin A. The results are shown in table 8.

TABLE 8

mμ moles added	of (^{3}H)-piericidin A/g of protein bound after number of washes down 0	1	2	3
1.3	0.65	0.37	0.30	0.28
2.6	1.18	0.56	0.42	0.35

E. The effect of other inhibitions on the binding of (^{3}H)-piericidin A mitochondria.

Beef heart mitochondria (1.0ml, 20mg of protein) were suspended in 0.25M sucrose and 50mM tris HCl, pH 7.6, containing 2% bovine serum albumen (25ml), at 30°C, and incubated for 8 minutes with ranges of concentrations of unlabelled piericidin A, rotenone, amytal and antimycin A. (^{3}H)-Piericidin A (0.5 mμmoles) was added in ethanolic solution, and the suspension incubated for a further 8 minutes. The mitochondria were collected and their radioactivity was measured as in the experiments described above.

The results are shown in Table 9.

TABLE 9

unlabelled piericidin A added mμmoles/g	(^{3}H)-piericidin A bound mμ moles/g	rotenone added mμ moles/g	(^{3}H)-piericidin A bound mμ moles/g	amytal added mM	(^{3}H)-piericidin A bound mμmoles/g	antimycin A added mg/g	(^{3}H)-piericidin A bound mμmoles/g
0	16.2	0	16.2	0	16.3	0	16.2
7.15	14.6	5.5	14.1	1.0	15.0	2.75	16.8
14.3	13.2	13.75	13.5	3.0	10.6	5.5	16.1
35.8	10.1	27.5	11.5	5.0	8.5		
71.5	8.2	55	8.4				

The Recovery of (^{3}H)-piericidin A from Mitochondria (performed in conjunction with Mr. A.J.Sweetman)

Beef heart mitochondria (25ml) (equivalent to 0.5g of mitochondrial protein) were incubated at 30°C for 8 minutes with 0.25M-sucrose/50mM-tris-HCl (100ml), pH 7.6, containing 2% w/v bovine serum albumen (BSA). (^{3}H)-piericidin A (0.02 μ mole), 1.13 μC) in ethanol (0.1 ml) was added. The mitochondria were collected by centrifugation at 30,000 xg and then washed in sucrose-tris-BSA medium. The pellets were suspended in sucrose-tris-medium (100ml). An aliquot (0.1ml) of this suspension was added to scintillating fluid (10ml), and the radioactivity measured in a Packard Tri-Carb liquid scintillation spectrometer.

Radioactivity (observed-background) = 1,800 c.p.m.
(uncorrected for self absorption)

$$\text{Apparent total of piericidin A bound} = \frac{1{,}800 \times 10^3 \times 10^9}{3.7 \times 10^{10} \times 60 \times 56.5}$$

= 14.4 mμ moles

The mitochondria were again collected by centrifugation. The pellets were extracted with acetone (100ml for 5 mins) by a process of suspension and centrifugation yielding an oily solid (78mg.), which in

TEXT BOUND INTO

THE SPINE

turn yielded a yellow oil (23.3mg) on elution from a short silicic acid (Mallinckrodt) column with acetone. The oil was dissolved in 50% v/v methanol/chloroform mixture (4ml) and an aliquot (0.01ml) counted as before.

Radioactivity (observed-background) = 4,750 c.p.m.
(uncorrected for self absorption)

Apparent total of piericidin A bound = $\frac{4,750 \times 400 \times 10^9}{3.7 \times 10^{10} \times 60 \times 56.5}$ mμ moles

= 15.1 mμ moles

The remaining material was examined chromatographically (t.l.c. system C) for phospholipids with reference to samples of authentic materials. The results are shown in table 10, together with the differential radioactivity of the chromatogram, measured after extracting its fraction with chloroform.

TABLE 10

Chromatogram Fractions	R_f	0-0.1	0.1-0.2	0.2-0.3	0.3-0.4	0.4-0.5	0.5-0.6	0.6-0.7	0.7-0.8	0.8-0.9	0.9-1.0
Radioactivity Content	c.p.m	0	0	0	0	0	0	0	0	270,000	1,310,000
Chromatogram Spots	R_f			-		0.41	0.55	0.64			0.93
Phosphatidyl Inositol	R_f			0.27							
Phosphatidyl Choline	R_f					0.41					
Phosphatidyl Ethanolamine	R_f						0.55				
Cardiolipin	R_f							0.64			
Piericidin A	R_f										0.93

Chromatography of Mitochondrial Extract

The fractions containing the radioactivity (Rf0.8-1.0) were combined to yield a yellow oil (2.3mg). The R_f value of the radioactive component of this oil was the same as piericidin A on two other chromoatographic systems.

t.l.c. (System A) R_f =0.22

t.l.c. (System B) R_f = 0.57

No spectroscopic absorption attributable to piericidin A (eg. λmax at 232 and 239 nm) was detected in any of the above preparations.

Attempted detection of Interaction between Piericidin A and Amino Acids

A sealed ampoule containing 0.25mM-(^{3}H)-piericidin A in methanol (50 µl , 0.705 µ C) and 0.1ML-lysine in water (50 µl) together with a 3:1 v/v methanol/water mixture (5ml) was heated at 85°C for 12 hours. At the end of this the solution was recovered and the solvent removed on the rotary evaporator. The residue was examined chromatographically for radioactivity (T.L.C. system A).

The experiment was repeated using other amino acids in place of lysine and also with no amino acid.

The results are shown in table 11.

Under the chromatographic conditions used it was shown independently that all the amino acids remained at the origin. They were detected using a 2% ninhydrin spray.

TABLE 11

Amino Acid	Radioactivity of Chloroform Extracts (c.p.m.) R_f 0 - 0.1	R_f 0.2 - 0.33	% recovery* in these two extracts	Radioactivity Ratio R_f 0-01: R_f 0.2 - 0.33
L-Lysine	77,000	1,230,000	83	16
L-Lysine HCl	95,000	1,190,000	82	12.5
L-Glutamine	90,000	1,270,000	87	14
L-Glutamic Acid	87,000	1,190,000	82	13.5
L-Cystine	78,000	1,150,000	79	14.5
L-Cysteine	114,000	1,140,000	80	10
L-Cysteine HCl	380,000	830,000	78	2.2
None	93,000	1,250,000	86	13.5

* 0.705 uC of (^{3}H)-piericidin A were used for each experiment (1,560,000 c.p.m.)

Spectroscopic Investigation of Piericidin A and Ubiquinone as Charge-Transfer Donors

The absorption maxima of the following compounds were measured in freshly prepared Dichloromethane solution in the range 240-777nm.

	λ Max.nm	ϵ Max
Tetracyanoethylene (TCNE)	266 276	15,000 13,400
2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)	285 387	11,600 955
Ubiquinone (UQ)	275 400	13,600 480
Hericidin A (PA)	267	5,300
Tetramethoxy-1,4-benzoquinone (TMQ)	298 408	17,600 330

The absorption maxima of equimolar mixtures of TCNE or DDQ with UQ, PA or TMQ were measured as before. No absorptions unattributable to the individual compounds of each pair were detected.

Charge transfer colours were obtained with the following equimolar mixtures in dichloromethane, in order to evidence the suitability of the conditions.

(i) Hexamethylbenzene with TCNE or DDQ (blue)

(ii) Benzidine with TCNE or DDQ (mauve)

THE 2,4-DINITROPHENYLHYDRAZONES OF METHYL PYRUVATE

Diazomethane in ethereal solution was prepared from p-tolylsulphonylmethylnitrosamide as described by Vogel[213]

Methyl pyruvate was prepared in 85% yield by the reaction of diazomethane with pyruvic acid (167)

B.Pt.. 133-137°C (lit (219) 134-137°C)

IR Spectrum

ν (thin film) cm^{-1}

2960 (med.st.)	1275 (st.)
1735 (v.st.)	1195 (st.)
1440 (st.)	1140 (v.st.)
1362 (st.)	1002 (st.)
1307 (st.)	838 (med.st.)
	740 (med.st.)

^{1}H NMR Spectrum

τ ($CDCl_3$) 6.14 (S) 3H, 7.54 (S) 3H

Methyl Pyruvate - α2,4-dinitrophenylhydrazone was prepared in 90% yield, as yellow cystals, according to the general method of Smith and Jones (168)

M.Pt.. 186 - 187°C (lit. (148) 142 - 144°C)
(lit. (149) 186.5 - 187.5°C)
(lit. (150) 184 - 185°C)

Chromatographic Homogeneity

T.C.L. (System D) One component, R_f = 0.43, yellow

UV Spectrum

λ max (methanol) 361 nm (ϵ_{MAX} = 17,400)

IR Spectrum (lit(150) 361 nm (ϵ_{MAX} = 17,200))

ν (nujol mull) cm^{-1}

3150 (med.st.)	1338 (st.)
3090 (w)	1303 "
1700 (st.)	1205 "
1618 "	1167 "
1592 "	1140 "
1574 "	1102 "
1510 "	844 "
	744 "
1415 "	744 "

-4.1 (broad s.)1H, 0.90(d,J=2 Hz)1H,
1.8(m)2H, 6.06(S)3H,7.69(S)3H

Mass Spectrum m/e (I)

282	(79)	152	(15)
266	(1)	135	(6)
251	(1)	122	(6)
222	(8)	43	(100)
181	(15)		

Peaks due to metastable transitions

$282^{+} \longrightarrow 181^{+} + 101 @ 115.82$
$181^{+} \longrightarrow 122^{+} + 59 @ 82.24$

Analysis:

Found C, 42.61; H, 3.67; N, 19.69%

$C_{10}H_{10}N_4O_6$ requires C, 42.55; H, 3.55; N, 19.86%

The addition of 1N Sodium hydroxide solution to a methanolc solution of the above compound produced no colour change.

Methyl Pyruvate - β,2,4-dinitrophenylhydrazone

Methyl Pyruvate-α, 2,4-dinitrophenylhydrazone (50mg), dissolved in methanol (150 ml) acidified with sulphurylchloride (2 drops), was exposed to sunlight for one month in a silica flask. Chromatographic examination of the resulting solution (T.L.C.System D) showed the presence of starting material (R_f = 0.43,yellow) and a new compound (R_f = 0.16, yellow) as spots of similar intensity. The residue left on evaporation of methanol was separated into its two components by elution from a silicic acid (Mallinckrodt) column with hexane containing an increasing proportion of benzene. The new compound was further purified by preparative T.L.C., followed by recrystallisation from methanol,yielding orange crystals (7mg, 14% yield).

M.Pt. 164.5 - 165.5°C

T.L.C. (System D) One component, R_f = 0.16, yellow

UV Spectrum

λ_{max} (methanol) 347 nm (ϵ_{MAX} = 22,800)

IR Spectrum

ν (nujol mull) cm^{-1}

3310	(med.st.)	1262	(st)
3080	(med.st.)	1225	(")
1730	(st.)	1200	"
1619	"	1136	"
1592	"	1102	"
1501	"	835	"
1342	"	737	"
1310	"	717	"

^{1}H NMR Spectrum

($CDCl_3$, 1%) -1.0 (broad S),1H,0.83(d,J=2 Hz)1H, 1.7(m)2H, 6.09(S)3H,7.71(S)3H

Mass Spectrum m/e (I)

282	(65)	152	(15)
266	(1)	135	(6)
251	(5)	122	(6)
222	(8)	43	(100)
181	(15)		

Peaks due to metastable transitions:

$282^+ \longrightarrow 181^+ + 101$ @ 115.82

$181^+ \longrightarrow 122^+ + 59$ @ 82.24

The addition of 1N Sodium Hydroxide solution to a methanolic solution of the above compound produced a red colour, dischargable on acidification.

Acetone-2,4-dinitrophenylhydrazone

This dinitrophenylhydrazone was prepared in 90% yield, as orange crystals, according to the general method of Smith and Jones (168).

M.Pt. 126°C sharp (lit (168) 126°C)

UV Spectrum

λ_{max} (methanol) 356nm

The addition of 1N Sodium Hydroxide solution to a methanolc solution of the above compound produced a red colour, dischargable on acidification.

THE SYNTHESIS OF 4,6-DIMETHOXY-2,3-DIMETHYL-5-HYDROXYPYRIDINE AND OF 4,6-DIMETHOXY-2,5-DIMETHYL-3-HYDROXYPYRIDINE

2,3-dimethylpyridine-N-oxide was prepared in 84% yield from 2,3-lutidine by a method analogous to that described for pyridine-1-oxide by Ochiai (169)

B.Pt 90°/0.5 mm Hg (lit (170) 118°C/4 mm. Hg)

UV Spectrum

λmax (methanol) 259 nm (ϵ_{MAX} 9,300)

IR Spectrum

ν (thin film) 1265 cm^{-1} (v.st.)

2,3-dimethyl-4-nitropyridine-1-oxide was prepared in 75% yield from 2,3-dimethylpyridine-1-oxide by a method analagous to that described for 4-nitropyridine-1-oxide by Ochiai (169). The reaction temperature was 90°C instead of 130°C.

Yellow, cystalline M.Pt. 92-93°C (lit(170) 91.5-93°C)

UV Spectrum

λ max (methanol) 241nm (ϵ_{MAX} 9,800)

316 nm (ϵ_{MAX} 9,900)

IR Spectrum

ν (nujol mull) 1278 cm^{-1} (v.st.)

^{1}H NMR Spectrum

τ ($CDCl_3$) 1.82 (d, J = 6.9 Hz) 1H,

2.33 (d, J = 6.9 Hz) 1H,

7.52 (S) 6H

4-nitropyridine-1-oxide was prepared in 88% yield from pyridine-1-oxide according to the method of Ochiai (169)

Yellow, crystalline, M.Pt. 161.5 - 162.5°C)

(lit (169) 159°)

UV Spectrum

λ max (methanol) 233 nm (ϵ_{MAX} 9,300)

328 nm (ϵ_{MAX} 14,100)

IR Spectrum

ν (nujol mull) 1265 cm^{-1} (v.st.)

^{1}H NMR Spectrum

τ ($CDCl_3$) 1.79 (m, A_2B_2 system with J_{AB} = 8.0 Hz)

2,4-dichloropyridine was prepared in 49% yield by the action of sulphuryl chloride on 4-nitropyridine-1-oxide, at 110°C in a sealed tube, as described by Kolder and den Hertog (172).

B.Pt.	75°C/1.5 mm Hg	(lit. (172) 73-75°/1.5 mm Hg)
UV Spectrum λ max(methanol)		258 nm, 265 nm, 272 nm
1H NMR Spectrum τ ($CDCl_3$)		1.63 (d,5.5 Hz)1H, 2.60(m)1H, 2.70 (double d., J=5.5 Hz and 1.7 Hz)1H.

2,4-dichloropyridine was prepared in 48% yield by the action of redistilled phosphorus oxychloride under reflux, in place of sulphuryl chloride, on 4-nitropyridine-1-oxide, in a modification of the experiment described immediately above. The product was identical to that described above.

4,6-dichloro-2,3-dimethylpyridine

2,3-dimethyl-4-nitro-pyridine-1-oxide (50g) was dissolved in redistilled phosphorus oxychloride (200 ml.) On raising the temperature to 65°C a vigorous reaction commenced, oxides of nitrogen being evolved. When this abated the mixture was heated under reflux (circa 110°C) for three hours. On cooling, the excess phosphorus oxychloride was removed by distillation. The reaction mixture was poured onto ice, adjusted to pH 8 with ammonium hydroxide solution, and extracted three times with chloroform. Evaporation of solvent from the combined dried extracts yielded an oil (53g), which was examined directly by vapour phase chromatography (using a Perkin Elmer F11 instrument, having a flame ionisation detector, and a 50ft. LAC Capillary Column at 160°C, with all gas inlet pressures at 25 psi)

This showed the presence of three products, A,B and C, referred to in their order of elution, in apparent proportions of 13, 5 and 1. *(corresponding to apparent gravimetric yields of 72,28 and 6%). Preliminary separation was by elution from a Woelm cationic alumina column with dry benzene, the order of elution being C, B, A. Components A and B were further purified by preparative T.L.C. (System E A, R_f = 0.5; B, R_f = 0.75). Component C was recrystallised from methanol. The purity of all three samples was shown by vapour phase chromatography to be more than 98%

* The proportion of component C produced became negligable (i.e. < 1% gravimetric yield) when the experiment was repeated using 20g of 2,3-dimethyl-4-nitropyridine-N-oxide and 250 ml of phosphorus oxychloride, other conditions remaining unchanged.

A, 4 6-dichloro-2,3-dimethylpyridine

White, crystalline, M.Pt. 46 - 47°C

UV Spectrum

λmax (methanol) 272 nm

1 H NMR Spectrum

τ($CDCl_3$) 2.80 (S)1H, 7.49(S)3H, 7.71(S)3H

Mass Spectrum m/e (I)

179 (11)	(M+)
177 (60)	(M+)
175 (100)	(M+)
162 (4)	
160 (6)	
140 (15)	
139 (17)	
123 (22)	
109 (46)	
99 (26)	

Analysis

Found: C,47.67; H,4.15; N,8.09; Cl,40.24%

$C_7H_7N\,Cl_2$ requires: C,47.75; H,3.98; N,7.96; Cl,40.34%

B, 4-chloro-2-chloromethyl-3-methylpyridine

Colourless oil, B.Pt.. 62°C/0.3 mm. Hg

UV Spectrum

λmax (methanol) 267 nm

^{1}H NMR Spectrum

τ ($CDCl_3$) 1.80 (d, J = 5.2)H,
2.81 (d, J = 5.2)1H
5.35 (S)2H, 7.55 (S)3H

Mass Spectrum m/e (I)

179 (6) (M+)
177 (30) (M+)
175 (43) (M+)
142 (35)
140 (100)
105 (21)

Peak due to Metastable Transition

$175^+ \longrightarrow 140^+ + 35$ @ 112.00

Analysis

Found: C, 47.87; H, 4.33; N, 7.66 %

$C_7H_7N\,Cl_2$ requires: C, 47.75; H, 3.98; N, 7.96 %

C, 2-cyano-4,6-dichloro-3-methylpyridine

White, crystalline, M.Pt. 94-95°C

UV Spectrum

λmax (methanol) 284, 293 nm

IR Spectrum

ν (nujol mull) 3070 (med.st.), 2240 (w) cm^{-1}

^{1}H NMR Spectrum τ ($CDCl_3$) 2.46 (S)1H, 7.46(S)3H.

Mass Spectrum m/e (I)

190 (13) (M+)
188 (66) (M+)
186 (100) (M+)
163 (2)
161 (14)
159 (20)
151 (41)
150 (31)
126(11)
124 (28)
115 (13) 99(16)

Peaks due to metastable transitions

$188^+ \longrightarrow 161^+ + 27$ @ 137.87
$186^+ \longrightarrow 159^+ + 27$ @ 135.92
$188^+ \longrightarrow 151^+ + 37$ @ 121.28
$186^+ \longrightarrow 151^+ + 35$ @ 122.59
$188^+ \longrightarrow 150^+ + 38$ @ 119.69
$186^+ \longrightarrow 150^+ + 36$ @ 120.97

Analysis:

Found: C, 44.80 H, 2.21 N, 14.71 Cl, 37.64 %

$C_7H_7N_2Cl_2$ requires: C, 44.92; H, 2.14; N, 14.97; Cl, 37.97 %

4,6-dimethoxy-2,3-dimethylpyridine

Sodium methoxide (40g) dissolved in dry, redistilled, methanol(200ml) was added to a mixture of 4,6-dichloro-2,3-dimethyl-pyridine (14.5g) together with 4-chloro-2-chloromethyl-3-methylpyridine (5.5g) in an autoclave, reaction being at 130°C for three hours. On cooling, excess methanol was removed by distillation, and water (100 ml) was added. The solution was extracted three times with chloroform, the combined extracts after drying and removal of solvent, yielding an oil (18g). Short path distillation (see page 129) of this at room temperature, and a pressure of 0.01 mm Hg, yielded an oily distillate (12.1g) and a solide residue (5.8g).

The oil was separated into two components by elution from a Woelm neutral alumina column. The first component, A (7.2)g, 52% partial yield) was eluted with benzene, the second, B (4.3g) (82% partial yield) with a 50% v/v benzene/ethyl acetate mixture.

Their homogeneity was established by t.l.c. (System D, A* R_f = 0.80; B, R_f = 0.23)

The solid distillation residue C was recrystallised from methanol (4.3g, 34% partial yield).

* This compound produced no colour with the Dragendorff reagent.

A, 4,6-dimethoxy-2,3-dimethylpyridine

Colourless crystals	M.Pt31-32°C
UV Spectrum λ_{max} (methanol)	268 nm
IR Spectrum ν (thin film) cm^{-1}	3010 (med), 2950 (med), 1599 (v.st), 1479 (v.st.), 1457 (st.), 1405 (med.), 1387 (med.), 1373 (st.), 1347 (v.st.) 1268 (med.), 1208 (v.st.), 1185 (med.) 1155 (v.st.), 1227 (v.st.), 1064 (st.) 998 (med.), 904 (med.), 817 (st.) 756 (med).
^{1}H NMR Spectrum τ ($CDCl_3$)	3.97(S)1H, 6.14(S)3H, 6.24(S)3H, 7.64(S)3H, 7.98(S)3H
Mass Spectrum	See figure 44 of the mass spectral appendix

Peak due to metastable transition

$167^+ \longrightarrow 166^+ + 1$ @ 165.01

Analysis:

Found:	C,64.51; H 7.78; N, 8.57%
$C_9H_{13}O_2N$ requires:	C, 64.67; H, 7.78; N, 8.38%

B 4-methoxy-2-methoxymethyl-3-methylpyridine

M.Pt. 36-37°C

IR - Spectrum (thin film) cm^{-1} 2940 (med.), 1585 (v.st.), 1480(st.) 1440 (st.), 1377 (st.), 1291 (v.st.), 1192 (med.), 1130 (st.), 1087 (v.st.), 1014, 984, 956, 915 (all.med), 828-818 (st.)

^1H NMR Spectrum $\tau(CDCl_3)$ 1.72 (d,J= 6.0 Hz)1H, 3.37 (d,J = 6.0 Hz) 1H, 5.47 (s) 2H, 6.19 (s) 3H, 6.62 (s)3H, 7.81 (s)3H.

Mass Spectrum See figure 44 of the mass spectral appendix

Peak due to metastable transition $137^+ \longrightarrow 122^+ + 15$ @ 108.64

C 4-hydroxy-1,2,3-trimethyl-6-pyridone

White, crystalline, M.Pt. 261-262°C

Chromatographic Homogenity

TLC System F	One Spot, R_f = 0.05
System C	One Spot, R_f = 0.90

UV Spectrum

	λ_{MAX} nm	ϵ_{MAX}
[methanol: water, 3:1, methanol: .1NNaOH, 3:1]	286	6,700
(methanol: .1NHCl, 3:1)	272	7,200

IR Spectrum

ν(nujol mull) cm^{-1} 3410 (med), 3110 (med.), 1658 (shoulder), 1638 (v.st.), 1550 (st.), 1412 (st.) 1253 (med.), 1238 (st.), 1187(st.), 1131 (st.),1040 (st.), 1007(med), 970, 940 (med.), 817, 767 (st.)

^{1}H NMR Spectra

τ ($CDCl_3$, saturated)	4.23 (s)1H, 6.25 (s)3H, 7.72 (s)3H, 8.07 (s)3H
τ ($CDCl_3$, with T.A.I)	- 1.9 (s)1H, 3.57 (s)0.4H, 3.78 (s)0.6H, 6.14 (s)1.2H, 6.17(s)1.8H, 7.56 (s)1.2H, 7.66(s)1.8H, 7.88 (s)1.2H, 7.99(s)1.8H,

MassSpectrum

m/e	I	
153	100	(M^+)
138	15	
125	9	
124	9	
110	38	

Peak due to metastable transition

$153^+ \longrightarrow 138^+ + 15$ @ 124.47

Analysis:

Found:	C,63.05;	H,7.13;	N,9.15%
$C_8H_{11}O_2N$ requires:	C,62.75;	H,7.09;	N,9.15%

The Stability of 4-hydroxy-1,2,3-trimethyl-6-pyridone to conditions under which aromatic methyl ethers cleave.

A 48% Hydrobromic Acid

4-Hydroxy-1,2,3-trimethyl-6-pyridone (500mg) in 48% w/v hydrobromic acid (25 ml) was heated under reflux and the product extracted according to the method described for the hydrolysis of 2-(1-n-hexyl)-4-, and 2-(1-n-hexyl)-6- methoxypyridines (page 187). Starting material (420 mg) was recovered, identified by its melting point (259-261°C) and infra red spectrum. No other material was isolated.

B Hydriodic Acid (d = 1.7)

4-Hydroxy-1,2,3-trimethyl-6-pyridone (500mg) was heated under reflux with red phosphorus (4g), acetic anhydride (10 ml) and hydriodic acid, d = 1.7,

(10 ml), analogously to the method described in organic syntheses (179) for the hydrolysis of N-methyl-4-hydroxy-3-methoxyphenylalanine. The product was isolated by extraction with chloroform instead of by precipitation. Starting material (397 mg) was recovered as the only isolated material, identified by its melting point (259-261°C) and infra red spectrum.

C Boron Tribromide

Boron tribromide (2.5g) in dichloromethane (10ml) was slowly added to a slurry of 4-hyrdoxy-1,2,3-trimethyl-6-pyridone (500mg) in dichloromethane (20ml) at - 80°C, producing a solution. This was allowed to warm up to room temperature and stirred for 16 hours, whereupon it was shaken with water, the pH of which was adjusted to 6 with ammonium hydroxide solution. The dichloromethane layer was separated and combined with two further chloroform extracts. These extracts were washed with water, dried, and the solvent evaporated yielding starting material (474mg), identified by its melting point (260-262°C) and infra red spectrum.

4,6-dimethoxy-2,3-dimethyl-5-nitropyridine was prepared from 4,6-dimethoxy-2,3-dimethylpyridine in 51% yield by a method analogous to that described for 2,4-dimethoxy-3-nitropyridine by Johnson, Katritzky et al [180]. The product was purified by elution from a Woelm neutral alumina column with dry benzene.

Yellow, crystalline, M.Pt. 18-20°C

Chromatographic homogeneity

TLC System G — One spot R_f = 0.85
(this compound produced no colour with the Dragendorff reagent)

UV Spectrum — λ_{max} = 275 nm

IR Spectrum
ν (thin film) cm^{-1} — 2960, 2930, 2855 (all med.) 1598 (v.st.), 1585 (sh), (1538 (v.st.), 1462, 1407, 1391, 1372 (all st.), 1343, 1279, 1259 (all med.), 1212 (st.) 1123, 1013 (all st.), 910 (med), 793 (st.)

^{1}H NMR Spectrum
τ ($CDCl_3$) — 6.03 (s)3H, 6.13(s)3H, 7.59 (s)3H, 7.88 (s)3H

Mass Spectrum

m/e	I	
212	100	(M^+)
182	29	
165	17	
151	17	
137	29	

Analysis: by accurate mass determination of molecular ion of mass spectrum.

Found: 212.0786

$C_9H_{12}N_2O_4$ requires: 212.0797

A second compound (24 mg, equivalent to 0.5% yield) was obtained from a later column eluent, and was purified by preparative TLC (System G R_f = 0.70)
(this compound produced no colour with the Dragendorff reagent)

Colourless crystals M.Pt. 72-74°C

IR Spectrum
ν (nujol mull) cm^{-1} — 2240 (med), 1608 (v.st.), 1565 (st.), 1428 (st.), 1275 (med.), 1220 (v.st.) 1189, 1165, 1102, 1050 (all st.), 1012 948, 895 (all med.), 851 (st.)

^{1}H NMR Spectrum

τ ($CDCl_3$) 3.68 (s)1H, 6.10(s)3H, 6.14(s)3H, 7.72(s)3H

This compound was not investigated further, but the spectroscopic data is consistent with the structure 2-cyano-4,6-dimethoxy-3-methylpyridine.

5-amino-4,6-dimethoxy-2,3-dimethylpyridine

Raney nickel (100 mg) was added to 4,6-dimethoxy-2,3-dimethyl-5-nitropyridine (600 mg) in methanol (50 ml) and the mixture hydrogenated at one atmosphere pressure. After three hours the catalyst was removed magnetically, and the solvent by distillation on the rotary evaporator, yielding an oil (510 mg). Short path distillation (see page 129) of this at room temperature and 0.01 mm Hg gave colourless crystals (490 mg) (95% yield), darkening on exposure to light.

M. Pt. 31 - 33°C

Chromatographic homogeneity

TLC System E — One Spot R_f = 0.55

TLC System H — One Spot R_f = 0.72 (sensitive to enol spray)

UV Spectra

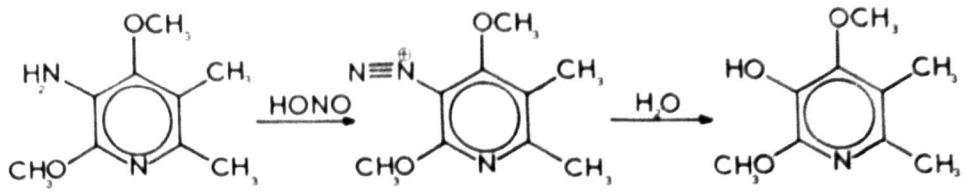
(methanol)	λ_{max} nm	λ_{max} nm
(methanol plus 2 drops 1N NaOH)	240	289
(methanol plus 2 drops 1N HCl)	240	289
	272	319

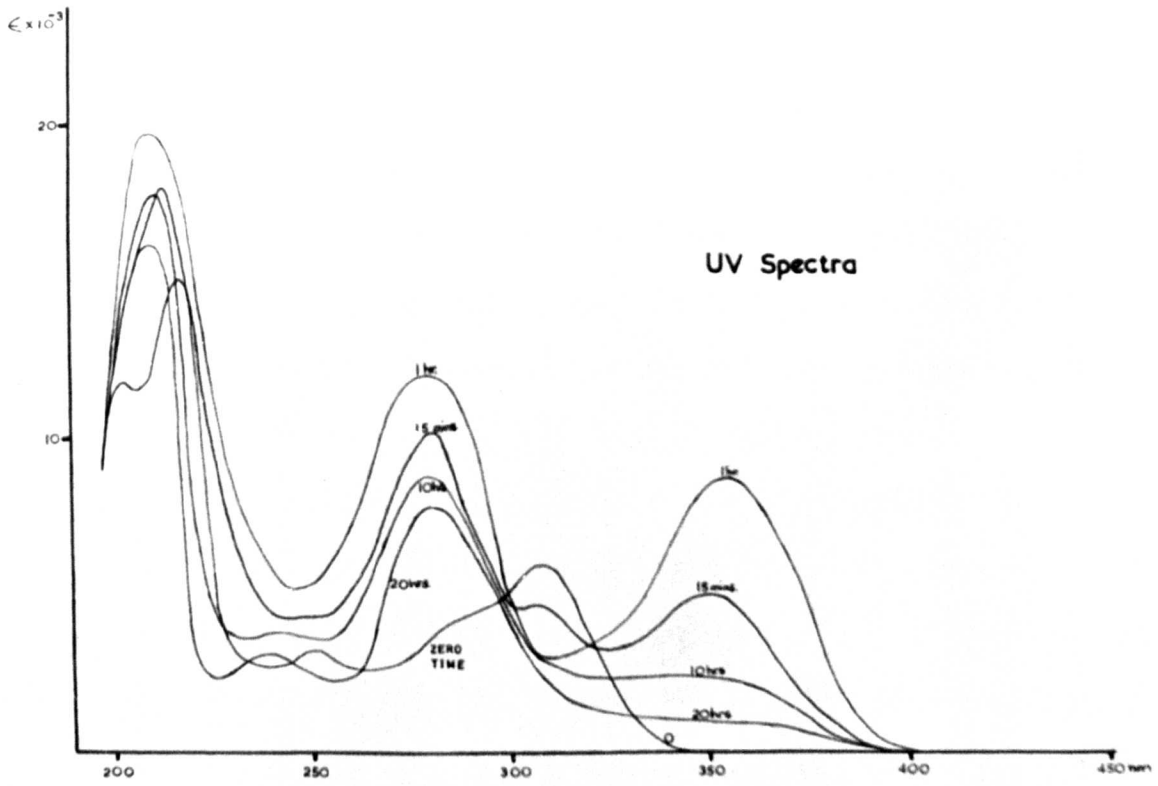
IR Spectrum

ν (thin film) cm^{-1} 3460, 3360, 2950 (all st.), 2865 (med.) 1598, 1470 (all v.st.), 1427, 1414, 1381, 1349, 1283, 1258, 1197, 1160, 1090, 1029, 1007 (all st.) 891, 873 (med.)

FIGURE 39

SPECTROPHOTOMETRIC COURSE OF REACTION





Test for diazonium salt using resorcinol impregnated paper

Time	Result
O	−ve
15mins.	+ve
1hr.	+ve
10hrs	faintly +ve
20hrs	−ve

^{1}H NMR Spectra

τ ($CDCl_3$) 6.09 (s)3H, 6.28 (s)3H, 7.73(s)3H, 7.93(s)3H, amino protons not visible

τ (D_6DMSO) 5.72(s)2H, slowly exchanged by added D_2O, 6.17(s)3H, 6.37(s)3H, 7.77(s)3H, 7.96(s)3H.

Mass Spectrum

m/e	I	
182	100	(M^+)
167	12	
153	3	
152	3	
149	3	
139	42	
124	6	

Peaks due to metastable transitions

$182^+ \longrightarrow 167^+ + 15$ @ 153.24
$182^+ \longrightarrow 139^+ + 43$ @ 106.16

Analysis:

Found: C, 59.63; H,7.75; N,15.25 %

$C_9H_{14}O_2N_2$ requires: C, 59.34; H, 7.69; N, 15.38%

4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine

Sodium nitrite (96mg) in water (5 ml) was added to 5-amino-4,6-dimethoxy-2,3-dimethylpyridine (250 mg) in 1M-sulphuric acid (250 ml), initially at 0°C. The reaction was followed spectrophotometrically, and using a spot test for diazonium salts (Resorcinol impregnated paper), and was complete after 20 hours at room temperature (see figure 39). The solution was adjusted to pH7 with potassium hydroxide solution, and extracted three times with chloroform. The combined extracts, after drying and removal of solvent on the rotary evaporator, yielded an oil (130 mg.) This was purified by TLC (System E R_f = 0.50) followed by short path distillation, at room temperature and 0.01 mm Hg, yielding long colourless birefringent crystals (120 mg, 48% yield)

M.Pt. 66°C (Sharp)

Chromatographic homogeneity

TLC Systhem E	One Spot R_f = 0.50
TLC System H	One Spot R_f = 0.22 (sensitive to Enol Spray)

UV Spectra

	λ_{max} nm	ϵ_{max}
(methanol)	281	6,000
(methanol:water,3:1)	281	6,000
(methanol:.1NNaOH,3:1)	247	7,400
	297	7,100
(methanol:.1NHCl,3:1)	291	7,800

IR Spectra

ν(thin film) cm^{-1} 3420 (broad st.), 2975 (st.), 1605 (v.st.) 1463, 1413 (all v.st.), 1383, 1348, 1298 1247, 1192(all st.), 1120 (v.st.), 1033, 1006, 903, 869 (st.)

ν($CHCl_3$) cm^{-1} 3550, 1610, 1460, 1295, 1115 (all v.st) 1003, 902 (all st.)

^1H NMR Spectra

τ($CDCl_3$) 6.08(s)3H, 6.10(s)3H, 7.70(s)3H, 7.93(s)3H, hydroxy proton not visible

τ($CDCl_3$ plus TAI) 1.06(s)1H, 6.11(s)3H, 6.15(s)3H, 7.65(s)3H, 7.92(s)3H

τ(HMPA) 5-7 (v.broad flat signal exchanged by addition of D_2O - presumably hydroxy proton) 6.17(s)3H, 6.23(s)3H, 7.81(s)3H, 8.03(s)3H

Mass Spectrum See figure 45 of the mass spectral appendix

No peaks due to metastable transitions detected.

Analysis:

Found:	C, 59.03; H,6.97; N, 7.96%
$C_9H_{13}NO_3$ requires:	C, 59.02; H,7.10; N, 7.65%

Note

Using a method based on that for the preparation of 5-hydroxy-2-methoxypyridine from 5-amino-2-methoxypyridine, but using M/10 sulphuric acid, by Adams and Govindachari [182], no phenolic product was isolated from 5-amino-4,6-dimethoxy-2,3-dimethylpyridine.

Indeed, at the point where the temperature was raised to 80°C, a red compound was suddenly formed, having a strong infrared absorption at:

$\nu(CHCl_3)$ 2160 cm^{-1}

This may have been a diazonium compound, or an azo-dye. On boiling with 1M-sulphuric acid for 3 hours, in the presence of a trace of copper sulphate, no change could be detected. The compound was therefore not investigated further.

2,5-dimethylpyridine-1-oxide was prepared in 80% yield from 2,5-lutidine by a method analogous to that described for pyridine-1-oxide by Ochiai[169].

B. Pt. 95°C/0.5 mm Hg	(lit.[171] 132-134°C/1mm Hg) (lit.[170] 86°C/0.3 mm Hg)
UV Spectrum λ_{max} (methanol)	259 nm (ϵ_{MAX} = 9,100)
IR Spectrum	
ν(thin film) cm^{-1}	1275 (v.st.)
^1H NMR Spectrum	
$\tau(CDCl_3)$	1.88(s)1H, 2.9(m)2H, 7.52(s)3H, 7.73(s)3H

2,5-dimethyl-4-nitropyridine-1-oxide was prepared in 77% yield from 2,5-dimethylpyridine-1-oxide by a method analogous to that described for 4-nitropyridine-1-oxide by Ochiai[169]. The reaction temperature was 90°C instead of 130°C.

Yellow crystalline, M.Pt. 152-153°C (lit.[170] 151-152°C) (lit.[171] 174-175°C)

UV Spectrum (methanol)	λ_{max} nm	ϵ_{max}
	239	7,950
	329	11,200

IR Spectrum

ν(nujol mull) cm^{-1} 1298 (v.st)

^{1}H NMR Spectrum

τ($CDCl_3$) 1.80(s)1H, 1.97(s)1H, 7.42(s)3H, 7.47(s)3H

4,6-dimethoxy-2,5-dimethylpyridine

A 2,5-dimethyl-4-nitropyridine-1-oxide (20g) was treated with redistilled phosphorus oxychloride (250 ml) in the same manner described earlier for the case of 2,3-dimethyl-4-nitropyridine-1-oxide (page 161).

B The crude product (20g) of the above reaction was reacted with sodium methoxide, in a manner similar to that described earlier (page 164) in an analogous experiment, yielding an oil (17.5g). Short path distillation of this, at room temperature and a pressure of 0.01 mm Hg, yielded an oily distillate (12g) and a solid residue (5g).

The oil was separated into two components by elution from a Woelm neutral alumina column, monitored by TLC (System E). Elution with benzene gave low melting point crystals (7.9g, 39% yield over two stages), A, a second eomponent, B, (2.4g, 12% yield over two stages) being eluted later with a 50% v/v benzene/ethyl acetate mixture. The solid distillation

residue was recrystalised from methanol; C, (3.7g, 20% yield over two stages).

A. 4,6-dimethoxy-2,5-dimethylpyridine

Colourless, crystalline, M.Pt. 21-22°C

Chromatographic homogeneity

TLC System E — One Spot R_f = 0.84
(This compound produced no colour with the Dragendorff reagent and was detected with iodine vapour)

UV Spectrum

λ_{max}(methanol) — 266 nm (ϵ_{MAX} = 4,400)

IR Spectrum

ν(thin film) cm^{-1} — 3010, 2950, 2870 (all med.) 1600-1588 (v.st.), 1485, 1455, 1365, 1338, 1293, 1219, 1181, 1167 (all st.), 1142 (v. st.), 1043, 979, 815 (all st.)

^{1}H NMR Spectrum

$\tau(CDCl_3)$ — 3.67(s)1H, 6.09(s)3H, 6.21(s)3H, 7.61(s)3H, 8.01(s)3H.

Mass Spectrum — See figure 44 of the Mass spectral appendix.

B 4-methoxy-2-methoxymethyl-5-methylpyridine

Colourless oil, solidifying in ice. M.Pt. 0-3°C

Chromatographic homogeneity

TLC System E — One Spot, R_f = 0.12

IR Spectrum

ν(thin film) cm^{-1} — 2935, 2865, 2830 (all med.) 1603 (v.st.), 1575, 1502, 1467, 1388, 1377, 1311, 1250, 1198, 1157, 1110 (all st.) 1039 (v.st.) 953, 853, 753, 733 (all St.)

^{1}H NMR Spectrum

$\tau(CDCl_3)$ — 1.86(s)1H, 3.13(s)1H, 5.50(s)2H, 6.15(s)3H, 6.57(s)3H, 7.88(s)3H

Mass Spectrum — See figure 44 of the mass spectral appendix.

Analysis:

Found: C, 64.39; H, 7.67; N, 8.88%

$C_9H_{13}NO_2$ requires: C, 64.67; H, 7.78; N, 8.38%

C. 4-hydroxy-1,3,6-trimethyl-2-pyridone

White, crystalline. M.Pt. 185–187°C

UV Spectra	λ_{max} nm	ϵ_{max}
{methanol; methanol: water, 3:1; methanol: .1NNaOH, 3:1}	292	7,300
(methanol: .1NHCl, 3:1)	277	7,800

IR Spectrum

ν (nujol mull) cm^{-1} — 3280 (w), 1630 (v.st.) 1563, 1339 (med), 1276 (st), 1210(med), 1145 (v.st.), 1042, 950, 906, 765 (all med)

^1H NMR Spectrum

τ ($CDCl_3$) — 3.3(broad s)1H, (exchanged by addition of D_2O), 4.13(s)1H, 6.19(s)3H , 7.64(s)3H, 8.03(s)3H.

Mass Spectrum

m/e	I	
153	100	(M^+)
138	39	
124	17	
123	28	
110	13	
94	19	

Peak due to metastable transition

$153^+ \longrightarrow 138^+ + 15$ @ 124.47

Analysis

Found: C, 62.97; H, 7.22; N, 9.53%

$C_8H_{11}O_2N$ requires: C, 62.75; H, 7.19; N, 9.15%

4,6-dimethoxy-2,5-dimethyl-3-nitropyridine was prepared from 4,6-dimethoxy-2,5-dimethylpyridine in 51% yield by a method analogous to that described by Johnson, Katritzky et al[180] for 2,4-dimethoxy-3-nitropyridine. The product was purified by elution from a Woelm neutral alumina column with dry benzene.

Yellow, crystalline, M.Pt. 69-71°C

Chromatographic homogeneity

TLC System H — One spot, R_f = 0.85

UV Spectrum

λ_{max}(methanol) — 269 nm (ϵ_{MAX} = 5,300)

IR Spectrum

ν(nujol mull) cm^{-1} — 1580, 1525, 1342, 1195, 1132, 1077 995, 916 (all st.) 874 (med), 800 (st.) 782, 739, 707 (all med.)

^{1}H NMR Spectrum

τ($CDCl_3$) — 6.04(s)3H, 6.14(s)3H, 7.57(s)3H, 7.90(s)3H.

Mass Spectrum

m/e	I	
212	100	(M^+)
197	16	
195	20	
182	44	
167	18	
165	24	
140	14	
137	24	

Peak due to metastable transition

$212^+ \longrightarrow 182^+ + 30$ @ 156.25

Analysis:

Found:	C, 50.77;	H, 5.66;	N, 13.06%
$C_9H_{12}N_2O_4$ requires:	C, 50.94;	H, 5.66;	N, 13.21%

3-amino-4,6-dimethoxy-2,3-dimethylpyridine was prepared in 96% yield, by hydrogenation of the corresponding 3-nitro compound, according to the method described earlier for the preparation of 5-amino-4,6-dimethoxy-2,3-dimethylpyridine (see page 170)

Colourless, crystals, M.Pt. 28-29°C

Chromatographic homogeneity

TLC System E — One spot, R_f = 0.65 (sensitive to enol spray)

IR Spectrum

ν (thin film) cm^{-1} — 3440, 3355, 2950, 2855 (all med.) 1590, 1460, 1412, 1384 1344, 1276, 1199, 1133, 1076, 1022, 995 (all st.) 904, ,865 (med).

^1H NMR Spectra

τ ($CDCl_3$) — 6.14(s)3H, 6.26(s)3H, 6.7(broad s),2H, exchanged by addition of D_2O, 7.71(s) 3H, 7.93(s)3H

τ (D_6DMSO) — 5.87(s)2H, exchanged by addition of D_2O. 6.27(s)3H, 6.34(s)3H, 7.78(s)3H, 8.01(s)3H

Mass Spectrum

m/e	I
182	100
181	36
167	30
152	39
139	29
124	12

Analysis:

Found:	C, 59.33;	H, 7.74;	N, 15.74%
$C_9H_{14}N_2O_2$ requires:	C, 59.34;	H, 7.69;	N, 15.38%

4,6-dimethoxy-2,5-dimethyl-3-hydroxypyridine was prepared in 53% yield, via the diazonium salt from the corresponding 3-amino compound, according to the method described earlier for the preparation of 4,6-dimethoxy-2, 3-dimethyl-5-hyrdoxypyridine (page 171).

Colourless, crystalline, M.Pt. 94-95°C

Chromatographic homogeneity

TLC System H	One Spot, R_f = 0.2 (sensitive to enol spray)

UV Spectra

	λ_{max} nm	ϵ_{max}
(methanol: water, 3:1)	287	5,900
(methanol: .1NNaOH, 3:1)	295	7,100
(methanol: .1NHCl, 3:1)	309	6,400
	238	7,400

IR Spectra

ν (nujol mull) cm^{-1}	3370 (broad st.), 1609, 1413, 1349 (all st.), 1307 (med.), 1233, 1193, 1134, 1084 (all st.), 1014, 915, 965 (med).
ν ($CHCl_3$) cm^{-1}	3550, 1609, 1460, 1409, 1349, 1132, 1080 (all st.)

^1H NMR Spectra

τ ($CDCl_3$)	6.12(s)3H, 6.20(s)3H, 7.64(s)3H, 7.91(s)3H.
τ ($CDCl_3$ plus TAI)	1.01(s)1H, 6.08(s)3H, 6.18(s)3H, 7.69(s)3H, 7.92(s)3H
τ (HMPA)	6.15(s)3H, 6.23(s)3H, 7.71(s)3H, 8.04(s)3H 5-7 (very broad flat signal exchanged by addition of D_2O)

Mass Spectrum

See figure 45 of the mass spectral appendix

No peaks due to metastable transitions detected.

Analysis:

Found:	C, 59.34; H, 6.96; N, 8.02 %
$C_9H_{13}NO_3$ requires:	C, 59.02; H, 7.10; N, 7.65%

THE SYNTHESIS OF COMPOUNDS TESTED FOR THEIR INHIBITION OF NADH-LINKED OXIDATION

2-(1-n-hexyl)pyridine

2-picolyl lithium (9g) in ether was prepared according to the method of Organic Syntheses [184]. 1-Bromopentane (14g) in ether (50ml) was slowly added at 0°C. After 15 minutes, when the red colour of picolyl lithium was discharged, water (20ml), followed by hydrochloric acid (38% w/v, 20ml), was slowly added. The pH of the mixture was adjusted to 8 with sodium carbonate solution. The ethereal layer was separated, washed with water and dried, yielding an oil (14.5g). On distillation this yielded a clear colourless oil of 2-(1-n-hexyl)pyridine, B. Pt. 90-92°/6mm, (11.2g, 76% yield), (lit.[220] B.Pt. 87°/6mm)

UV Spectrum	λ_{max} nm	ϵ_{max}
(methanol)	265	3,150
	262	3,500
	269	2,600

^{1}H NMR Spectrum

τ (CCl_4) 1.56 (double d, J=4.7Hz and 2.0Hz)1H, 2.51 (double t, Jt = 7.5Hz and J_d = 2.0 Hz)1H, 3.0 (m., and double d., superimposed)2H, 7.28 (t, J= 7.8Hz)2H, 8.66 (m)8H, 9.12(t,J= 6Hz)3H

Mass Spectrum — See figure 42 of the mass spectral appendix.

2-(1-n-hexyl)pyridine-1-oxide

2-(1-n-hexyl)pyridine (7g) and peracetic acid in acetic acid (35% w/v, 50 ml) were mixed carefully at 0°C. The temperature was slowly raised to 80°C, and maintained at such. After 16 hours the excess acetic acid was removed on the rotary evaporator and the residue diluted with an equal quantity of water. After adjusting the pH to 8 withsodium

carbonate solution, the liquid was extracted with chloroform. The dried extract, after removal of solvent, yielded an oil (7.1 g, 92%) which was distilled (94-96°/5 mm).

UV Spectrum

λ_{max} (methanol)	261 nm (ϵ_{MAX} = 10,200)

IR Spedtrum

ν (thin film)	1245 cm^{-1} (v.st.)

^{1}H NMR Spectrum

τ ($CDCl_3$)	1.70 (m)1H, 2.75(m)3H, 7.03(t, J= 7.4Hz)2H, 8.62(m)8H, 9.11(t,J = 6Hz)3H

Note

After several attempts, starting material was recovered quantitatively on treating 2-(1-n-hexyl)pyridine with hydrogen peroxide(100 volume) and acetic acid, according to the method of Ochiai[169] for the preparation of pyridine-1-oxide from pyridine.

Attempted Nitration of 2-(1-n-hexyl)pyridine-1-oxide

A. Following the procedure of Ochiai[169] for the nitration of pyridine-1-oxide with nitric acid (d = 1.48) in sulphuric acid, only starting material (72%) was recovered from 2-(1-n-hexyl) pyridine-1-oxide, being identified from its infra red spectrum. Unidentified, water soluble, highly coloured products were also produced.

On the substitution of fuming nitric acid for nitric acid (d = 1.48) in the same procedure, the reaction became violent and no starting material was recovered. Water soluble, highly coloured materials were again produced, but were not investigated.

B (i) 2-(1-n-hexyl)pyridine-1-oxide fluroborate

2-(1-n-hexyl)pyridine-1-oxide (5g) was dissolved in aqueous fluoroboric

acid (40% w/v, 20ml). The solution was extracted with chloroform three times. The combined extracts were dried, firstly over anhydrous sodium sulphate and secondly over molecular sieves (type 3A). Chloroform was removed by distillation, yielding a colourless oil of 2-(1-n-hexyl) pyridine-1-oxide fluoroborate (7.4g, quantitative yield).

IR Spectrum

ν(thin film) 1060 cm^{-1} (broad v.st.)

(ii) Attempted nitration with nitronium tetra fluoroborate

Nitroniumtetrafluoroborate (1.7g) in dry redistilled acetonitrile (10 ml) was slowly added to 2-(1-n-hexyl)pyridine-1-oxide fluoroborate (3g) in dry redistilled acetonitrile (10ml) at room temperature in a dry box. The addition was exothermic, and the temperature was not allowed to rise above 30°C. After stirring for 12 hours, the solution was poured onto ice, adjusted to pH 8 with ammonium hydroxide, and extracted three times with chloroform. The combined extracts, after drying, and removal of solvent by distillation, yielded 2-(1-n-hexyl)pyridine-1-oxide (2g, quantitative recovery), identified from its infra red spectrum.

The experiment was repeated, using nitromethane in place of acetonitrile, with the same result.

Reaction of 2-(1-n-hexyl)pyridine-1-oxide with Phosphorus Oxychloride

2-(1-n-hexyl)pyridine-1-oxide (5g) and redistilled phosphorus oxychloride were carefully mixed at 0°C, the process being exothermic. The temperature of the mixture was slowly raised, and it was heated under reflux (c.110°C). After 12 hours, excess phosphorus oxychloride was removed by distillation, and the mixture poured onto ice. After adjusting the pH to 8 with ammonium hydroxide solution, the liquid was extracted

with chloroform three times. The combined extracts were dried, and the solvent removed on the rotary evaporator, yielding a dark oil (4.8g), which was examined directly by vapour phase chromatography (using a Perkin Elmer F11 instrument, having a flame ionisation detector, and a 50ft LAC Capillary Column, at 180°C, with all gas inlet pressures 25 psi). This showed the presence of two products, A and B, together with starting material, in apparent proportions, respectively, of 23, 18 and 10 (corresponding to apparent gravimetric yields of 43, 34 and 19%). Product B was eluted from a Woelm neutral alumina column with benzene, followed later by product A. 2-(1-n-hexyl)pyridine-1-oxide was only eluted after products A and B, with 10% v/v methanol in benzene, and was identified from its infra red spectrum.

A. 4-chloro-2-(1-n-hexyl)pyridine

An oil.

UV Spectrum	λ_{max} nm	ϵ_{max}
(methanol)	261	3,300
	269	2,700

IR Spectrum

ν (thin film) cm^{-1} 3050(w), 2960, 2930, 2860, 1577, 1555, 1468, 1393, 1101, 823, 706 (all st.)

^{1}H NMR Spectrum

τ ($CDCl_3$) 1.39 (d, J = 5.0 Hz)1H, 2.7 (m,s. and d. superimposed)2H, 7.16 (t, J = 7.7 Hz)2H, 8.63(m)8H, 9.10(t, J = 6Hz)3H.

B 6-chloro-2-(1-n-hexyl)pyridine

An Oil.

UV Spectrum

λ_{max} (methanol)	268 nm (ϵ_{MAX} = 3,500)

IR Spectrum

ν (thin film)cm^{-1}	3060(w), 2960, 2930, 2860(st.) 1587, 1560, 1439, 1410 (st), 1380(w), 1163, 1140, 1101, 989, 789 (st.)

^{1}H NMR Spectrum

τ ($CDCl_3$)	2.31 (t, J = 7.7 Hz)1H, 2.76 (d, J = 7.7 Hz)1H, 2.82 (d, J = 7.7Hz)1H, 7.19 (t, J = 7.7 Hz)2H, 8.64 (m)8H, 9.11 (t, J = 6 Hz)3H.

2-methyl-5-ethylpyridine-1-oxide was prepared in 80% yield from 2-methyl-5-ethylpyridine by a method analogous to that described for pyridine -1-oxide by Ochiai[169].

M. Pt. 79-80°C	(lit.[221] 80°C)

IR Spectrum

ν (thin film)	1265 cm^{-1} (v.st.)

^{1}H NMR Spectrum

τ ($CDCl_3$)	1.81(s)1H, 2.05(s)1H, 7.06(q,J= 7.0Hz)2H, 7.51(s)3H, 8.71(t,J= 7.0 Hz)3H

Interaction of pyridine-1-oxides and phosphorus oxychloride at 33°C : an investigation by ^{31}P NMR Spectroscopy

(Performed in conjunction with Dr. K. Dillon)

2-(1-n-hexyl)pyridine-1-oxide (I,table 12) and 2-methyl-5-ethylpyridine-1-oxide(II,table 12) used in this experiment were dried by passage through molecular sieves (type 3A). Phosphorus oxychloride ($POCl_3$, table 12) and dichloroethane (S,table 12) were redistilled, and manipulation was in a dry box.

The compounds were mixed at room temperature in the volumetric proportions shown in table 12. The ^{31}P NMR spectra of the mixtures are also shown in table 12.

Hydrolysis of the mixtures containing pyridine-1-oxides on ice, within 1 hour of mixing, followed by extraction of the hydrolysate with chloroform, resulted in essentially quantitative recovery of the pyridine-1-oxides. They were identified by their IR spectra.

TABLE 12

Volumetric Proportions I	II	$POCl_3$	S	^{31}P NMR signals in p.p.m.relative to 85% phosphoric acid
0	0	1	1	-2.2 st.
1	0	1	1	-3.95 (st); + 3.2(med.); +8.3(w)
0	1	1	1	-3.95(st); + 3.9 (med); +8.75(w)
0	4	1	2.5	+4.05(st); +5.05(w); +10.05(w)

2 - (1-n-hexyl)-4-methoxypyridine

4-chloro-2-(1-n-hexyl)pyridine (150 mg) and sodium methoxide (250mg) in methanol (5ml) were heated in a sealed ampoule at 140°C. After 16 hours the mixture was recovered, and the methanol removed by distillation. The residue was extracted with hot chloroform, and the extract washed with water. On drying the solution and removing the chloroform by distillation an oil was obtained. After short path distillation at room temperature and a pressure of 0.01 mm Hg, 2-(1-n-hexyl)-4-methoxypyridine (110mg, 75% yield), an oil, was recovered.

Purity was ascertained by vapour phase chromatography.

UV Spectrum	
λ_{max} (methanol)	247 nm(shoulder) (ϵ_{MAX}= 1,900)
IR Spectrum	
ν(thin film) cm^{-1}	3010(w), 2960, 2930, 2860 (st) 1595, 1567, 1482, 1460, 1308, 1195, 1160, 1042(all st.) 993, 825, 810 (w)
^{1}H NMR Spectrum	
τ ($CDCl_3$)	1.70 (d, J = 5Hz)1H, 3.4 (s and d superimposed)2H, 6.21(s)3H, 7.27 (t, J = 7.7Hz)2H, 8.66 (m)8H, 9.11 (t, J = 6Hz)3H
Mass Spectrum	See figure 42 in the mass spectral appendix.

2-(1-n-hexyl)-6-methoxypyridine, an oil, was prepared in 75% yield from 6-chloro-2-(1-n-hexyl)pyridine in a manner analogous to that described in the experiment immediately above.

UV Spectrum

λ_{max}(methanol) 273 nm (ϵ_{MAX}= 3,400)

IR Spectrum

ν (thin film) cm^{-1} 3060(w), 2960, 2930, 2860(st.) 1598, 1578, 1466, 1438, 1414, 1340 1317 (all st.), 1279 (med), 1188 (med), 1148 (st.), 1101, 1073 (med) 1038, 986, 795, 735 (st.)

^{1}H NMR Spectrum

τ ($CDCl_3$) 2.56 (t, J = 7.7 Hz)1H
3.34 (d, J = 7.7 Hz)1H
3.49 (d, J = 7.7 Hz)1H
6.11 (s)3H, 7.33 (t, J = 7.7 Hz)2H,
8.66 (m)8H, 9.12(t, J = 6 Hz)3H

Mass Spectrum See figure 42 in the mass spectral appendix.

2-(1-n-hexyl)-4-hydroxypyridine

2-(1-n-hexyl)-4-methoxypyridine (100 mg) in 48% w/v hydrobromic acid (5 ml) was heated under refulx. After 16 hours, the pH of the solution was adjusted to 8, and it was extracted with chloroform. The extract was dried, and the chloroform removed by distillation. Short path distillation of the residual oil, at room temperature and a pressure of 0.01 mm Hg, yielded 2-(1-n-hexyl)-4-hydroxypyridine (85 mg, 92% yield), as a waxy solid.

M. Pt. 24-28°C

UV Spectrum

λ_{max}(methanol) 256 nm (ϵ_{MAX}= 13,800)

IR Spectrum

ν (thin film) cm^{-1} 3220, 3050 (med.) 2930, 2855 (st.) 1628, 1515 (v.st.), 1385, 1231, 862(med),

^{1}H NMR Spectrum

τ ($CDCl_3$) -0.2 (broad s.)1H, exchanged by

the addition of D_2O, 2.35 (d,J = 6.9 Hz)1H, 3.6 (d^2 and s superimposed) 2H, 7.35 (t, J = 7.7Hz)2H, 8.72 (m)8H, 9.12 (t, J = Hz)3H

Mass Spectrum — See figure 42 in the mass spectral appendix

Analysis:

Found: C, 73.71; H, 9.66; N, 7.66%

$C_{11}H_{17}NO$ requires: C, 73.75; H, 9.50; N, 7.82%

6-(1-n-hexyl)-2-hydroxypyridine, a waxy solid, was prepared in 95% yield from 2-(1-n-hexyl)-6-methoxypyridine in a manner analogous to that described in the experiment immediately above.

M. Pt. 46-47°C

UV Spectrum

λ_{max}(methanol) — 306 nm (ϵ_{max} = 5,300)

IR Spectrum

ν (thin film) cm^{-1} — 3270, 3120 (med), 2920, 2850(st.) 1640(broad v.st.), 1547, 1468(st.) 1416, 1377, 1228, 1211 (med). 1165, 1000, 950, 798, 788 (st.)

^1H NMR Spectrum

τ ($CDCl_3$) — -3.1 (broad s)1H exchanged by addition of D_2O, 2.73 (double d. J = 6.9 and 8.8 Hz)1H, 3.70 (d, J = 8.8 Hz)1H, 4.05 (d, J = 6.9Hz)1H, 7.42 (t, J = 7.7 Hz), 8.72 (m)8H, 9.12 (t, J = 6 Hz)3H.

Mass Spectrum — See figure 42 of the mass spectral appendix.

Analysis

Found: C, 73.51; H, 9.48; N, 7.25%

$C_{11}H_{17}NO$ requires: C, 73.75; H, 9.50; N, 7.82%

Benzyl Vanillin

Benzyl chloride (12.5g) in methanol(50ml) was slowly added to the sodium salt of vanillin (17.5g) in methanol (100ml), and the mixture heated under reflux. After 16 hours, the solvent was removed by distillation, and the residue dissolved in chloroform and washed with water three times. Drying and evaporation of the chloroform solution yielded crude benzyl vanillin (14.5g, 60% yield). On acidification of the aqueous washings followed by a similar chloroform extraction, vanillin (6g, 40% recovery, M.pt. 80–81°C (lit.[222] 80–81°C) was obtained.

On elution from a Woelm neutral alumina column with benzene the crude product, mentioned above, yielded chromatographically pure benzyl vanillin as an oil.

Chromatographic Homogeneity

TLC System G — One spot, R_F = 0.40
(Vanillin on the same system has an R_f of Zero)

IR Spectrum

ν_{CO} (nujol) — 1680 cm^{-1}

ν_{CO} (nujol) Vanillin — 1658 cm^{-1}

^1H NMR Spectrum

τ ($CDCl_3$) — 0.05(s)1H, 2.55(m)7H, 2.94 (d, J = 8.6 Hz)1H, 4.74(s)2H, 6.06(s)3H.

Triphenyl-(1-n-hexyl)phosphonium bromide (Wittig Salt)

Triphenylphosphine (26.3g) and 1-bromopentane (18.5g) in toluene (100ml) were heated under reflux for 16 hours. After this time, the mixture was cooled and the precipitated Wittig salt isolated by filtration and dried under vacuum at 70°C. (Yield = 26.8g white, crystalline, 65%)
M.Pt. 165–166°C (lit.[223] 165–165.5°C)

Note

The use of benzene instead of toluene in the above reaction reduced the yield of the Wittig salt to zero. Presumably this is because the temperature of boiling benzene is not sufficient to activate the reaction.

Benzyl-(4-(1-n-hex-1-enyl)-2-methoxyphenyl)ether

Triphenyl-(1-n-hexyl) phosphonium bromide (4.1g) and potassium tertiary butoxide (1.1g) in dry ether (100 ml) were mixed together in a slurry generating an orange ylid. Benzyl vanillin (2.4 g) in dry ether (50ml) was added slowly, after which the mixture was heated under reflux for 24 hours.

After washing the cooled mixture with water, evaporation of the dried ether solution yielded an oil (2.3g). Short path distillation of this at room temperature gave benzyl -(4-(1-n-hex-1-enyl)-2-methoxphenyl) ether (1.9g, 64% yield) as an oil.

Chromatographic homogeneity

TLC System G — One Spot, R_f = 0.90

^{1}H NMR Spectrum

τ ($CDCl_3$) — 2.54(m)5H, 3.11(s)3H, 3.60(d, J = 12.0 Hz)1H, 4.38 (double t; d, J = 12.0 Hz; t, J = 6.9 Hz)1H, 4.82(s) 2H, 6.12(s)3H, 7.65(q,J =7Hz)2H, 8.5 (m)4H, 9.10(t, J = 6.5Hz)3H

4-(1-n-hexyl)-2-methoxyphenol

10% Palladium on carbon (10 mg) was added to benzyl-(4-(1-n-hex-1-enyl)-2-methoxyphenyl)ether (100 mg) in methanol (25ml), and the mixture hydrogenated at one atmosphere pressure.

After the theoretical quantity of hydrogen was absorbed (15½ ml over 30 minutes), further absorption ceased. The catalyst was removed

by filtration, and the solvent on the rotary evaporator. Short path distillation, at room temperature and a pressure of 0.01 mm Hg, of the residual oil gave 4-(1-n-hexyl)-2-methoxyphenol (70mg, quantitative yield).

Chromatographic homogeneity

TLC System G — One Spot, R_f = 0.10

^{1}H NMR Spectrum τ ($CDCl_3$) — 3.2 (m, AB system and singlet superimposed)3H, 4.4 (broad s)1H, exchanged by addition of D_2O, 6.14 (s)3H, 7.45 (t, J = 7.3 Hz)2H, 8.65(m)8H, 9.12 (t, J = 6.5Hz)3H

2-hydroxymethyl-5-methoxy-4-pyrone was prepared in 72% yield, by the action of dimethylsulphate on kojic acid, according to the method of Campbell et al [193]

M. Pt. 163-164°C (lit.[193] 161°C)

UV Spectrum	λ_{max} nm	ϵ_{max}
(methanol)	265	10,300
(methanol: water, 3:1)	268	10,200
(methanol: .1NNaOH, 3:1)	268	9,400
(methanol: .1NHCl, 3:1)	268	11,700

IR Spectrum

ν(nujol mull) cm^{-1} 3180 (v.broad, st.), 1635, 1582 1256, 1217, 1144, 1080, 1050, 998 949, 866, 838, 716 (all st.)

^{1}H NMR Spectrum

$\tau(D_2O)$ 1.85(s)1H, 3.35(s)1H, 5.41(s)2H, 6.16(s)3H.

4-hydroxy-2-hydroxymethyl-5-methoxypyridine was prepared in 85% yield by the action of ammonia on 2-hydroxymethyl-5-methoxy-4-pyrone, according to the method of Armit and Nolan[194]. The product was resublimed as white crystals.

M. Pt. 178-179°C (lit.[194] 173-175°C)

IR Spectrum

ν(nujol mull) cm^{-1} 3400-2500(v.broad,st.), 1621 (v.st.) 1540(st.), 1268, 1250, 1168, 1153, 1081, 1017, 855, 765, 717 (all st.)

UV Spectrum	λ_{max} nm	ϵ_{max}
(methanol)	273	11,300
(methanol: water, 3:1)	275	11,500
(methanol: .1NNaOH, 3:1)	250	8,500
	275 (shoulder)	4,700
(methanol: .1NHCl, 3:1)	245	5,150
	270	5,300

^{1}H NMR Spectra

τ (D_2O) 2.42(s)1H, 3.50(s)1H, 5.39(s)1H, 6.20(s)3H

τ (DMSO) 2.54(s)1H, 3.69(s)1H, 4.7 (broad s.)2H, exchanged by addition of D_2O, 5.58(s)2H, 6.28(s)3H.

Mass Spectrum

m/e	I	
155	73	(M^+)
154	100	
140	20	
139	17	
126	42	
111	32	

Peaks due to metastable transitions

$155^+ \longrightarrow 154^+$ + 1 @ 153.01

$155^+ \longrightarrow 140^+$ + 15 @ 126.45

2,6-dimethyl-4-nitropyridine-1-oxide was prepared in 62% yield over two stages from 2,6-dimethylpyridine by a method analogous to that described for 4-nitropyridine-1-oxide-1-oxide by Ochiai[169]

M.Pt. 163-164°C (lit.[169] 163°C)

UV Spectrum	λ_{max} nm	ϵ_{max}
(methanol)	239	9,600
	232	9,700

IR Spectrum

ν (nujol) 1280 cm^{-1} (v.st.)

^1H NMR Spectrum $\tau(CDCl_3)$ 1.90(s)2H, 7.40 (s)6H

4-benzyloxy-2,6-dimethylpyridine-1-oxide was prepared in 65% yield from 2,6-dimethyl-4-nitropyridine-1-oxide by a method analogous to that described for 4-benzyloxypyridine-1-oxide by Ochiai[195]

M.Pt. 135°C

UV Spectrum

λ_{MAX}(methanol) 262 nm (ϵ_{MAX}= 16,800)

IR Spectrum

ν (nujol) 1230 cm^{-1} (v.st.)

^1H NMR Spectrum

τ ($CDCl_3$) 2.65(s)5H, 3.26(s)2H, 4.98(s)2H, 7.50(s)6H

2,6-dimethyl-4-hydroxypyridine-1-oxide

5% palladium on carbon (30 mg) was added to 4-benzyloxy 2,6-dimethyl-pyridine-1-oxide (230 mg) in ethanol(10 ml) and the mixture hydrogenated at one atmosphere pressure, analogous to the standard conditions of Katritzky and Monro[196] for the reduction of pyridine-1-oxides.

After 24 hours, the catalyst was removed by filtration and the solvent by distillation, yielding white crystals of 2,6-dimethyl-4-hydroxypyridine-

-1-oxide (130 mg. 92% yield). These were purified by reprecipitation, from methanolic solution, in cychlohexane, since recrystallisation proved to be difficult.

M. Pt. 194-196°C

UV Spectrum

λ_{max}(methanol) 259 nm (ϵ_{MAX} = 12,800)

^{1}H NMR Spectrum

τ ($CDCl_3$) 3.35(s)2H, 7.56(s)6H

Note Prolonged hydrogenation under the above conditions did not affect the course of the reaction.

2-Methyl-4-nitropyridine-1-oxide was prepared in 65% yield over two stages from 2-methylpyridine by a method analogous to that described for 4-nitropyridine-1-oxide by Ochiai[169]

M. Pt. 157-159°C (lit.[169] 153-4°C)

UV Spectrum

λ_{max} (methanol) 234 nm (ϵ_{MAX} = 9,200)
329 nm (ϵ_{MAX} = 14,300)

IR Spectrum

ν(nujol) 1285 (st.) cm^{-1}

^{1}H NMR Spectrum

τ($CDCl_3$) 1.75 (d,J = 6.9 Hz)1H, 2.0 (m)2H, 7.47(s)3H

2-methyl-4-nitropyridine was prepared in 70% yield from 2-methyl-4-nitropyridine-1-oxide by a method analogous to that described for 4-nitropyridine by Ochiai[169]

M.Pt. 40-42°C (lit.[225a] 32-34°C) (lit.[225b] 42-45°C)

UV Spectrum (methanol)	λ_{max} nm	ϵ_{max}
	227	6,300
	295	2,000

^{1}H NMR Spectrum

τ($CDCl_3$) 1.33 (d,J = 4.8Hz)1H, 2.25(m)2H 7.32(s)3H

4-hydroxy-2-methylpyridine was prepared in 82% yield from 2-methyl-4-nitropyridine by a method analogous to that described for the preparation of 4-hydroxypyridine by den Hertog et al[197]. The time of the reaction was three days instead of 12 hours. Oxides of nitrogen were produced during the reaction.

M. Pt. 177°C (lit.[226] 174-176°C)

UV Spectrum	λ_{max}^{nm}	ϵ_{max}
(methanol)	256	13,800
(methanol plus 2 drops .1NNaOH)	234	9,600
(methanol plus 2 drops .1NHCl)	240	11,400
	265 (shoulder)	2,800

IR Spectrum

ν (nujol)cm^{-1} 3400,,3270(med.),1630(st.) 1535(st.) 1230, 1180, 1095, 1048, 942, 924, 891, 829, ,730 (all st.)

$\nu(CHCl_3)cm^{-1}$ 3240, 3030, 2940 (med) 1637, 1508 (v.st.), 1395, 1386(st.)

^{1}H NMR Spectrum

$\tau(CDCl_3)$ -1.6 (broad s)1H, 2.41 (d, J = 6.9Hz)1H, 3.7(m)2H, 7.69(s)3H

Mass Spectrum See figure 45 of the mass spectral appendix

Peak due to metastable transition $109^+ \longrightarrow 80^+ + 29$ @ 58.72

4-hydroxy-2-methylpyridine nitrate

2-methyl-4-nitropyridine (M.Pt. 40-42°C), exposed to the atmosphere for two months,was found to have a melting range of 161-165°C. White crystals of 4-hydroxy-2-methylpyridine nitrate were recovered in 92% gravimetric yield on recrystallisation from acetone.

Effect of dry heat (i) melting
(ii) violent decomposition: no oxides of nitrogen detected, strong smell typical of a pyridine base.

Test for nitrite and nitrate ions using sulphanilic acid and α-napthylamine,with the addition of zinc dust later as a reducing agent.

Nitrite Test: Negative

Nitrate Test: Positive (red colour of azo dye)

M. Pt. 164°C with effervescence (lit.[198] 161.5 - 162°C)

UV Spectrum (methanol)

λ_{max} nm		ϵ_{max}
238		9,900
243	(shoulder)	9,600
248	"	9,400
252	"	9,100
257	"	7,900

IR Spectrum

ν (nujol) cm^{-1}

3100 (med,), 1641, 1628, 1613 (v.st.) 1500, 1315, 1250, 1214, 1185, 1040, 995, 869, 838, 710 (st.)

^1H NMR Spectrum

τ (D_2O)

1.71 (d, J = 7.2Hz)1H, 2.9(m)2H, 7.37(s)3H

τ (DMSO)

1.52 (d, J = 7.2Hz)1H, 1.9 (broad s.)2H exchanged by addition of D_2O, 2.9(m)2H, Signal from methyl group obscured by solvent.

Mass Spectrum

m/e	I
109	100
94	5
80	9
70	22
46	21
39	8
30	8

Peak due to metastable transition

$109^+ \longrightarrow 80^+ + 29$ @ 58.72

Analysis:

Found:	C, 42.03%;	H, 4.74%;	N, 16.42%
$C_6H_8O_4N_2$ requires:	C, 41.86%;	H, 4.65%;	N, 16.29%

THE SYNTHESIS OF OTHER PYRIDINES AND EXPERIMENTS CONCERNED WITH THE SYNTHESIS OF PYRIDINES

3-methoxypyridine

Diazomethane (0.64)g in ether (100 ml) was slowly added to 3-hydroxypyridine (1.44g) in methanol (25ml) at 0°C. After 30 mins. the solvent was removed by distillation, and the residue purified by short path distillation. 3-methoxypyridine (0.9g, 55% yield) was obtained as the distillate. The involatile residue (0.7g) was not identified.

B. Pt. 180°C	(lit.[228] 179°C)
UV Spectrum	
λ_{MAX}(methanol)	278 nm (ϵ_{MAX} = 4,000)
IR Spectrum	
ν (thin film)cm^{-1}	3010, 2960, 2840 (med.), 1576 1480, 1460, 1426, 1283, 1278, 1232, 1192, 1108, 1054, 1018, 797, 705 (all v.st.)
^{1}H NMR Spectrum	
τ ($CDCl_3$)	1.7m(2H), 2.8(m)2H, 6.18(s)3H.
Mass Spectrum	See figure 43 of the mass spectral appendix.

5-methoxy-2-methylpyridine was prepared in 52% yield from 5-hydroxy-2-methylpyridine by a method analogous to that described immediately above for the preparation of 3-methoxypyridine.

B. Pt. 190°C	(lit. [229] 188-189°C)
UV Spectrum	
λ_{max}(methanol)	284 nm (ϵ_{MAX} = 3,900)
IR Spectrum	
ν (thin film)cm^{-1}	3010, 2940, 2840, 1600 (all med.) 1575, 1499, 1486, 1463, 1441, 1390, 1268, 1241, 1213, 1181, 1134, 1120, 1030, 822, 722 (all st.)

^{1}H NMR Spectrum τ ($CDCl_3$) 1.8(m)1H, 2.95(m)2H, 6.20(s)3H, 7.51(s)3H.

Mass Spectrum See figure 44 of the mass spectral appendix.

4-methoxypyridine was prepared in 85% yield, from 4-chloropyridine, according to the method of Spinner and White[200].

B. Pt.. 86°/15mm (lit.[200] 107°/50mm)

M.Pt. 2-4°C (lit.[200] 4°C)

UV Spectrum

(methanol)	λ_{max}^{nm}	ϵ_{max}
	220	9,300
	245 (shoulder)	1,200)

IR Spectrum

ν (thin film)cm^{-1} 2960, 2840 (med,) 1597(v.st.),1570, 1501(st.), 1460, 1441, 1423 (all med.) 1289 (v.st.) 1212 (st.) 1029 (st.) 995 (med.), 820, 805(med.)

^{1}H NMR Spectrum τ($CDCl_3$) 1.63 (d, J = 5.0 Hz)2H, 3.25 (d, J = 5.0 Hz)2H, 6.23(s)3H

Mass Spectrum See figure 43 of the mass spectral appendix.

1-methyl-4-pyridone

The potassium salt of 4-hydroxypyridine (2.7g), methyl iodide (2.9g) and ethanol (5 ml) were heated at 100°C for 1.5 days in a sealed ampoule. After the removal of solvent from the recovered mixture, extraction with hot acetone yielded a semi-crystalline solid (1.8g). Short path distillation at 150°C and 0.01 mm Hg yielded colourless hygroscopic crystals (1.4g, 65% yield).

M. Pt. 90-94°C (lit.[230] 92-94°C)

UV Spectrum

λ_{MAX}(methanol) — 263 nm (ϵ_{MAX} = 18,200)

IR Spectrum

ν(thin film)cm^{-1} — 2960(m), 1648, 1550 (v.st.), 1440(m), 1397 (st) ,1364 (m), 1203 (v.st.), 1254, 1024 (m), 853 (st.)

^{1}H NMR Spectrum

τ($CDCl_3$) — 2.65 (d, J = 7.7Hz)2H, 3.67 (d,J = 7.7Hz)2H, 6.32(s)3H

Mass Spectrum — See figure 43 of the mass spectral appendix.

1-methyl-2-pyridone was prepared in 98% yield from 2-methoxypyridine by isomerisation in a sealed ampoule at 230°C for 2.5 days. The product was distilled at reduced pressure.

B.Pt. 90-92°C/1mm. Hg) (lit.[207b] 122-124°C/11 mm.Hg)

The distillate crystallised, with some difficulty in initiation, under nitrogen.

M. Pt. 28-30°C Colourless crystals (lit.[231] 30°)

UV Spectrum (methanol)

λ_{max} nm	ϵ_{max}
229	7,400
302	6,000

IR Spectrum ν(thin film)cm^{-1} — 3080, 3030, 2850 (med.), 1657, 1583 (v.st.), 1542,(st). 1470,1440, 1416, 1386 (med.), 1320 (st.) 1242 (med), 1156, 1064, 875, 843, 763, 726 (all st.)

^{1}H NMR Spectrum

τ($CDCl_3$) — 2.7(m, double d. and quartet of doublets superimposed) 3.43(double d, J = 9.5,1.4Hz)1H, 3.83(triplet of doublets, J = 6.5 1.4Hz)1H, 6.46(s)3H

Mass Spectrum — See figure 43 of the mass spectral appendix.

Attempted Hydroxylation of Pyridines with Hydrogen peroxide

A 2% v/v solution of pyridine in 100 volume hydrogen peroxide (50ml) was maintained at 50°C. After one week volatile material was removed on the rotary evaporator, the last traces being evaporated on the freeze dryer, yielding a semi-crystalline oil (0.54 g). This material was not appreciably soluble in acetone, methanol or other organic solvents, but was soluble in water, the solution colouring starch-iodide paper blue. Recrystallisation or reprecipitation of the material proved impracticable, but it was examined chromatographically.

TLC System F R_f = 0.05 (faint) and 0.60 (strong and tailing)
-detected using the enol spray

(R_f value of authentic 3-hydroxypyridine under these conditions = 0.20)

On maintaining the oil at 110°C under an air condenser for one week, a negligible quantity of material (17 mg) remained as an oily residue.

The experiment was performed concurrently with 2-,3- and 4-lutidine in place of pyridine, analogous results being obtained in each case.

EXPERIMENTAL COMPARISONS INVOLVING SUBSTITUENTS IN THE α, β AND γ POSITIONS OF PYRIDINES

^{1}H Nuclear magnetic resonance spectra of readily available pyridines

	τ ($CDCl_3$)
pyridine	1.43(m)2H, 2.40(m)1H, 2.82(m)2H
2-picoline	1.48(d, J = 5.1Hz)1H, 2.41(t, J = 6.9Hz)1H, 2.86(d, J = 6.9Hz)1H, 2.92(t, J = 6Hz)1H, 7.45(s)3H.
3-picoline	1.54(m)2H, 2.52(d, J = 7.4Hz)1H, 2.83(double d, J = 7.4 and 5.0 Hz)1H, 7.69(s)3H.
4-picoline	1.56(d, J = 5.0Hz)2H, 2.92(d, J = 5.0Hz)2H, 7.69(s)3H
2,3-lutidine	1.69(d, J = 5.1Hz)1H, 2.73 (d,J = 7.7Hz)1H, 3.02 (double d, J = 7.7 and 5.1Hz)1H, 7.52(s)3H, 7.75(s)3H.
2,4-lutidine	1.68 (d,J = 5.0Hz)1H, 3.08(s)1H, 3.14(d, J = 5.0Hz) 1H, 7.52(s)3H, 7.73(s)3H.
2,5-lutidine	1.69(s)1H, 2.64(d, J = 7.9Hz)1H, 2.99(d, J = 7.9Hz) 1H, 7.52(s)3H, 7.75(s)3H.
2,6-lutidine	2.71(t, J = 7.7Hz)1H, 3.21 (d,J = 7.7Hz)2H, 7.51(s)6H.
3,4-lutidine	1.72(s)1H, 1.75(d, J = 4.8Hz)1H, 3.03(d,J=4.8Hz) 1H, 7.79(s)6H.
3,5-lutidine	1.79(d, J = 2.0Hz)2H, 2.76(d, J = 2.0Hz)1H, 7.78(s)6H.
2,4,6-collidine	3.29(s)2H, 7.57(s)6H, 7.79(s)3H.
2,3,6-collidine	3.08(d, J = 7.9Hz)1H, 3.21(d, J = 7.9Hz)1H, 7.56(s)6H, 7.81(s)3H.
2-hydroxypyridine	2.63, (d, J = 6.5Hz)1H, 2.55(double d, J = 6.5 and 9.5Hz)1H, 3.45(d, J = 9.5Hz)1H, 3.75 (t, J = 6.5Hz)1H.
3-hydroxypyridine	1.72(s)1H, 1.94(d, J = 5.0Hz)1H, 2.75(m)2H.
4-hydroxypyridine	2.35 (d,J = 6.9Hz)2H, 3.57(d,J = 6.9Hz)2H

2-methoxypyridine	1.93(d, J = 5.0Hz)1H, 2.54(t, J = 7.5 Hz)1H, 3.23 (double d, J = 5.0 and 7.2 Hz)1H, 3.34 (d,J = 8.0Hz)1H, 6.12(s)3H.
2,6-dimethoxypyridine	2.56 (t,J = 8.2Hz)1H, 3.76(d, J = 8.2 Hz)2H, 6.13(s)6H.
2-hydroxy-3-methoxypyridine	3.02(d, J = 6.5Hz)1H, 3.31(d, J = 6.5Hz) 1H, 3.87(t, J = 6.5Hz)1H, 6.28(s)3H.
5-hydroxy-2-methylpyridine	1.87(s)1H, 2.86(m)2H, 7.53(s)3H.
3-hydroxy-2-hydroxymethyl-6-methylpyridine (saturated solution)	4.95(s), 7.50(s)
0,0-iso-propylidine pyridoxine	2.00(s)1H, 4.96(s)2H, 5.37(s)2H, 7.59(s) 3H, 8.43(s)6H.

The use of Hexamethyl Phosphoramide in the Proton NMR study of Hydroxypyridines

The ^{1}H NMR spectra of a series of compounds were recorded as their 10% solutions in redistilled hexamethylphosphoramide. The chemical shifts of protons attached to oxygen or nitrogen atoms in the spectra of these compounds, confirmed by their ready isotopic exchange with deuterium oxide, is shown below.

Compound	(HMPA) (Singlet)
2-hydroxypyridine	-2.3 (broad)
3-hydroxypyridine	-102 (sharp)
4-hydroxypyridine (anhydrous)	-0.6 (broad)
4-hydroxy-2-methylpyridine (anhydrous)	-0.4 (broad)
4-hydroxy-2-hydroxymethyl-5-methoxypyridine	+2.5 to + 4 (v.broad + flat)
5-hydroxy-2-methylpyridine	-0.67 (sharp)
2,3-dihydroxypyridine	0 to +2 (v.broad + flat)
4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine	+5 to +7 (v.broad + flat)

4,6-dimethoxy-2,5-dimethyl-3-hydroxypyridine	+ 5 to +7 (v.broad + flat)
4-methyl-2,6-di-t-butylphenol	+2 to +3 (v.broad + flat)
2-hydroxyquinoline	-2.5 (broad)
4-hydroxyquinoline	-2.8 (broad)
8-hydroxyquinoline	-0.5 (v.broad + flat)
Uracil	(1H at -1.6 / 1H at -1.7) (broad doublet)
2-naphthol (J.G.C)	-0.78 (sharp)
1-methyl-2-naphthol (J.G.C)	-0.65 (sharp)
1-hydroxymethyl-2-naphthol (J.G.C)	(1H at -0.57 (sharp) / 1H at +2.5 (broad))
2-hydroxy-1-naphthaldehyde (J.G.C)	-2.6 (broad)
Aniline	2H at 4.6 (broad)
Acetamide(slow isotopic exchange, $t_{\frac{1}{2}}^{33^\circ C}$ = 12 mins)	
	(1H at 1.95 (sharp) / 1H at 2.95 (sharp))
Benzamide(slow isotopic exchange, $t_{\frac{1}{2}}^{33^\circ C}$ = 2 mins)	
	(1H at 1.14 (sharp) / 1H at 2.25 (sharp))

J.G.C: compounds prepared and spectra recorded by Dr. J.G.Catterall.

The use of Trichloroacetylisocyanate in the ^{1}H NMR study of Hydroxypyridines

The ^{1}H NMR spectra of a series of compounds were recorded in 10% solution in deuterochloroform, or in the case of less soluble compounds as their saturated solutions. After the addition of one molar equivalent of trichloroacetylisocyanate (two molar equivalents in the case of a compound with two potentially reactive protons) the spectra were again recorded. The results are shown in table 13.

Deuterochloroform was chosen as the most suitable solvent after rejecting other possibilities on the following grounds.

Acetone and Carbon Tetrachloride: hydroxypyridines are relatively insoluble in these solvents.

Acetonitrile, nitromethane, dimethylformamide and prototropic solvents react with trichloracetylisocyanate.

Dimethylsulphoxide and Hexamethylphosphoramide: although both of these have good solvating properties towards hydroxypyridines, and neither appear to react with trichloracetylisocyanate, the solutions after the addition of trichloracetylisocyanate become viscous, producing a line broadening effect on the NMR signals to such an extent, in some cases, as to make the spectra uninterpretable.

KEY TO TABLE 13

Number	Compound
1	2-hydroxypyridine
2	3- "
3	4- "
4	4-hydroxy-2-methylpyridine
5	5- " -2- "
6	3-hydroxy-2-hydroxymethyl-6-methylpyridine
7	2,3-dihydroxypyridine
8	2-hydroxy-3-methoxypyridine
9	4-hydroxy-2-hydroxymethyl-5-methoxypyridine
10	2-(1-n-hexyl)-4-hydroxypyridine
11	6-(1-n-hexyl)-2-hydroxypyridine
12	4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine
13	4,6- " -2,5- " -3- "
14	Uracil
15	2,6-dihydroxy-4-methylpyridine

16 2-hydroxyquinoline

17 4- "

18 8- "

19 Piericidin A

20 Piericidin B

Symbols

CAT Spectrum composed with the aid of a computer of average transients (CAT).

* Compound insufficiently soluble to produce a spectrum, even with the aid of CAT.

Ⓧ The arrangements of substituents on the pyridine nucleus of these compounds is not certain.

TABLE 13

A = τ($CDCl_3$); B = τ($CDCl_3$ with TAI); C = A-B

Compound		Substituent Positions 2	3	4	5	6	-OH,-NH without TAI;	-NH with TAI
1	A	-	3.45	2.55	3.75	2.63	Not observed	-
	B	-	3.30	2145	3.50	1.64	-	-5.0
	C	-	0.15	0.10	0.25	0.99	-	-
2	A	1.72	-	2.75	2.75	1.94	2.3	-
	B	1.40	-	2.40	2.40	1.40	-	-1.1
	C	0.32	-	0.35	0.35	0.54	-	-
3	A	2.35	3.57	-	3.57	2.35	Not observed	-
CAT	B	1.35	2.60	-	2.60	1.35	-	-1.4
	C	1.00	0.97	-	0.97	1.00	-	-
4	A	7.67	3.78	-	3.71	2.45	1.9	-
	B	7.32	2.86	-	2.86	1.36	-	-2.21 (sharp)
	C	0.35	0.92	-	0.85	1.09	-	-
5	A	7.53	2.86	2.86	-	1.87	Not observed	-
	B	7.44	2.82	2.50	-	1.64	-	0.4
	C	0.09	0.04	0.36	-	0.23	-	-

Compound		Substituent Positions 2	3	4	5	6	-OH,-NH without TAI:	-NH with TAI
6	A	4.95	-	*	*	7.50	*	-
	B	4.55	-(2.27 or 2.71)			7.37	-	0.7 and 1.1
	C	0.40	-	-	-	0.13	-	-
7	A	-	-	*	*	*	*	-
	B	-	-	2.47	3.52	1.72	-	0.90 and -4.25 (both sharp)
	C	-	-	-	-	-	-	-
8	A	-	6.28	3.31	3.87	3.02	-3.5	-
	B	-	6.16	3.22	3.62	2.08	-	-4.85 (sharp)
	C	-	0.12	0.09	0.25	0.94	-	-
9	A	*	*	-	*	*	*	-
	B	4.66	2.64	-	6.02	1.61	-	0.8 and 3.3
	C	-	-	-	-	-	-	-
10	A	7.35	3.6	-	3.6	2.35	-0.2	-
	B	7.02	2.7	-	2.7	1.31	-	1.8
	C	0.33	0.9	-	0.9	1.04	-	-
11	A	7.42	4.05	2.73	3.70	-	-3.1	-
	B	7.07	3.83	2.64	3.55	-	-	-4.8
	C	0.35	0.22	0.09	0.15	-	-	-
12	A	7.70	7.93	6.10	-	6.08	Not observed	-
	B	7.65	7.92	6.16	-	6.11	-	1.06 (sharp)
	C	0.05	0.01	-0.05	-	-0.03	-	-
13	A	7.64	-	6.20	7.91	6.12	Not observed	-
	B	7.69	-	6.18	7.92	6.08	-	1.01 (sharp)
	C	-0.05	-	0.02	-0.01	0.04	-	-
14 & 15	A	-	-	*	*	-	*	-
	B	-	-	*	*	-	-	*
	C	Apart from their insolubility neither compound appeared to react with TAI even at 80°C						

Compound		Substituent Positions 2	3	4	5	6	-OH,-NH without TAI:	-NH with TAI
		2	3	4	others			
16	A	-	3.26	2.16	2.3-3.0		-2.6	-
	B	-	3.26	2.16	2.3-3.0		-	-2.6
	C	No reaction appeared to take place in this case even at 80°C						
17	A	*	*	-	*	*	*	-
	B	2.59	4.18	-	3.2-4.1		-	0.0
	C	-	-	-	-		-	-
18	A	1.20	(2.3-2.9)	1.86	2.3-2.9		Not observed	-
	B	1.07	(2.1-2.7)	1.81	2.1-2.7		-	0.1
	C	0.13	0.2	0.05	0.2		-	-
19 ⊗	A	6.08	6.18	6.63	7.91		Not observed	-
	B	6.04	6.18	6.60	7.94		-	0.93 and 1.77 (both sharp)
	C	0.04	zero	0.03	-0.03		-	-
20 ⊗	A	6.08	6.18	6.64	7.91		Not observed	-
	B	6.03	6.17	6.59	7.92		-	0.97(sharp)
	C	0.05	0.01	0.05	-0.01		-	-

An ultraviolet spectroscopic investigation of a series of hydroxypyridines and related compounds in neutral, acidic and basic solutions was undertaken. The solvents contained methanol in order to extend comparisons of these compounds to the pericidins, which are not sufficiently soluble in completely aqueous media to produce detectable ultraviolet absorptions.

Neutral Solvent[2d]. 75% methanol, 25% water (v/v)

Acidic Solvent[2d]. 75% methanol, 25% 0.1N Hydrochloric acid

Basic Solvent[2d]. 75% methanol, 25% 0.1N sodium hydroxide solution.

The results are presented in table 14.

TABLE 14

A) 2-Hydroxypyridines and Related Compounds

Compound	λ_{max}^{nm} (ϵ_{MAX})		
	Neutral Solvent	Acidic Solvent	Basic Solvent
2-hydroxypyridine	298(5,200)	294(5,300)	292(5,500)
1-methyl-2-pyridone	297(6,000)	294(5,000)	-
2-methoxypyridine	271(3,000)	295(5,200)	-
2-(1-n-hexyl)-6-hydroxypyridine	306(5,700)	300(5,900)	303(5,500)
2-(1-n-hexyl)-6-methoxypyridine	274(3,100)	301(5,600)	-

B) 3-hydroxypyridines and Related Compounds

Compound	λ_{max}^{nm} (ϵ_{MAX})		
	Neutral Solvent	Acidic Solvent	Basic Solvent
3-hydroxypyridine	277(4,200)	285(7,300) 224(4,000)	300(4,700) 238(11,100)
3-methoxypyridine	278(4,100)	286(7,500)	-
5-hydroxy-2-methylpyridine	283(4,100)	292(7,200)	303(4,800) 240(9,900)
5-methoxy-2-methylpyridine	284(3,900)	292(7,100)	-
4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine	281(6,000 226(4,800)	291(7,800)	297(7,100) 248(7,400)
4,6-dimethoxy-2,5-dimethyl-3-hydroxypyridine	287(5,900)	295(7,100)	307(6,400) 238(7,400)
2,4-dimethyl-3-hydroxy-6-methoxypyridine (HMO)	290(4,500)	306(7,200)	316(5,000)
Phenol	272(1,560)	271(1,440)	289(2,700)

(HMO): Compound prepared and spectra recorded by Mr. H.M.Okely.

c) 4-hydroxypyridines and related compounds

Compound	λ_{max}^{nm} (ϵ_{MAX})		
	Neutral Solvent	Acidic Solvent	Basic Solvent
4-hydroxypyridine	256(14,800)	234(9,900)	240(14,500) and shoulder 265(3,200)
1-methyl-4-pyridone	263(18,200)	240(13,000)	-
4-methoxypyridine	220(9,300) and shoulder 245(1,200)	235(9,700)	-
2-methyl-4-hydroxypyridine	256(13,800)	234(9,600)	240(11,400) and shoulder 265(2,800)
2-(1-n-hexyl)-4-hydroxy pyridine	256(13,500)	234(9,700)	239(12,100) and shoulder 265(2,800)
2-(1-n-hexyl)-4-methoxy pyridine	220(9,000) 247(1,700)	235(9,400)	-
4-hydroxy-2-hydroxymethyl-5-methoxypyridine	274(11,500)	245(5,100) 270(5,300)	250(8,500) and shoulder 270(4,900)
2-hydroxymethyl-5-methoxy-4-pyrone	267(10,200)	267(11,700)	267(9,400)
D) The Piericidins			
Piericidins A and B	267(5,600)	273(8,200)	Shoulder 270(3,200)
Octahydropiericidin A	267(5,600)	274(8,200) 233(5,600)	Shoulder 240(9,000) 270(2,700)
Piericidin A diacetate	278(6,500)	280(6,400)	Shoulder 270(3,000)

Thermal Stability of 4,6-dimethoxy-2,3-dimethylpyridine

A 4,6-dimethoxy-2,3-dimethylpyridine was heated at 180°C for 3 days in a sealed ampoule. 82% of the starting material was recovered by short path distillation at room temperature and 0.01 mm Hg after this time.

M. Pt. 31-32°C

The infra red spectra of the starting material and distillate were identical.

B After heating 4,6-dimethoxy-2,3-dimethylpyridine at 230°C for 3 days in a sealed ampoule, no starting material or volatile product was recovered. The solid residue, corresponding gravimetrically to quantitative isomerisation of the starting material, was not investigated further.

Thermal Stability of 2-methoxypyridine

A 2-methoxypyridine was heated at 180°C for 3 days in a sealed ampoule. 98% of the starting material was recovered by distillation after this time.

B.Pt. 90-92°/mm. Hg. (lit.[207b] 122-124°C/11mm. Hg)

The infra red spectra of starting material and distillate were identical.

B 2-methoxypyridine was converted in 98% yield to 1-methyl-2-pyridone on heating at 230°C for 2.5 days in a sealed ampoule. (see the preparation of 1-methyl-2-pyridone, page 201)

The base catalysed deuterium exchange of protons in the methyl groups of pyridines was investigated under three sets of conditions. The degree of exchange was calculated from a comparison of the integrated proton NMR spectra of the individual compounds before and after reaction, taking into account the estimated isotopic ratio of deuterium to hydrogen in themedium after reaction. An example of this calculation is shown below for the case of 2-methylpyridine. The results of this, and other experiments are shown in Table 15, together with the conditions, A, B or C used in each case, and the percentage of starting material recovered.

Experimental Conditions

A The pyridine base (100 mg) in 4N Sodium Deuteroxide in deuterium oxide (99.7% ^2H 5ml), was maintained at 100°C. After 7 days the pH of the solution was adjusted to 7 with deuterochloric acid, and the solution extracted with chloroform. The extract was dried, the solvent evaporated and the recovered material purified by short path distillation at room temperature and a pressure of 0.01 mm Hg.

B The pyridine base (500 mg) in 1N sodium deuteroxide in deuterium oxide (1.5 ml) was maintained at 180°C in a sealed ampoule for 1 day. The recovery of the reaction product was by the method described immediately above.

C The pyridine base (200 mg) in 4N sodium deuteroxide in deuterium oxide (4 ml), with redistilled dry dioxan (2ml), was maintained at 180°C in a sealed ampoule for 3 days. The reaction product was recovered by the method described above, except in the case of octahydropiericidin A which was recovered by preparative TLC (System A).

Calculation of deuterium exchange of protons in 2-methylpyridine subjected to the experimental conditions described in B, above.

τ ($CDCl_3$)	1.38(d)	2.40(t)	2.9(m)	7.46(s)
Assignment to position of substitution in pyridine ring	6	4	3 and 5	2
Ratio of peak areas before reaction * (±0.05)	0.97	1.00	1.93	3.02
Ration of peak areas after reaction* (±0.05)	0.61	1.00	1.65	0.39
% Incorporation of Deuterium	37(±10)	0(±10)	14(±5)	87(±3.3)
% Scrambling x	42(±11)	0(±11)	16(±5.5)	98(±3.7)

* measured gravimetrically on scale expanded spectra.

x % deuterium in medium after reaction

$$= \frac{(D) \times 100}{(D) + (H)} = \frac{\left[\frac{2 \times 1.5}{20} - \frac{3.3 \times 0.5}{93}\right] \times 100}{\left[\frac{2 \times 1.5}{20} - \frac{3.3 \times 0.5}{93}\right] + \left[\frac{3.3 \times 0.5}{93}\right]}$$

$$= 89\%$$

KEY TO TABLE 15

NUMBER	Compound
1	2-methylpyridine
2	3-methylpyridine
3	4-methylpyridine
4	5-methoxy-2-methylpyridine
5	5-hydroxy-2-methylpyridine(sodium salt)
6	4,6-dimethoxy-2,3-dimethylpyridine
7	4,6-dimethoxy-2,5-dimethylpyridine
8	4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine(sodium salt)
9	4,6-dimethoxy-2,5-dimethyl-3-hydroxypyridine(sodium salt)
10	Octahydropiericidin A

TABLE 15

Number of Compound	Conditions of Exchange	% Recovery of Starting Material	% Scrambling in pyridine ring positions				
			2	3	4	5	6
1	B	92	98%*	(16%)	R	(16%)	42%
2	B	96	(29%)	18%*	R	28%	(29%)
3	B	96	21%	R	103%*	R	21%
4	B	96	67%*	(28%)	(28%)	R	98%
5	B	86	95%*	R	R	(-OH)	96%
5	A	95	No isotopic exchange detected				
6	B	93	8%*	2%*	R	None	R
6	C	81	76%*	None*	R	29%	R
7	C	81	12%*	None	R	None*	R
8	C	59	56%*	5%*	R	(O-H)	R
9	C	52	50%*	(-OH)	R	None*	R
10	C	57	No isotopic exchange detected				

Experiment 10 was performed on 75 mg. of starting material.

R: A substituent whose NMR signal was used as a reference, on the assumption that no isotopic exchange had taken place on it.

( ): Scrambling percentages which are bracketted are of substituents which have NMR signals coincident with those of other substituents.

* Methyl substituents.

Exchange of phenolic protons (OH) was instantaneous.

As in the case of 2-methyl pyridine (see above), the error in the quoted % scrambling is estimated as $\pm$ 4% for methyl substituents and $\pm$ 12% for single proton substituents.

Colour Reactions

Dragendorff Reagent[215]

Application was as a spray on dried spots of 10% solutions of test compounds. The resultant colour was estimated after 15 minutes.

Blue:	diethylaniline
Red:	2-hydrazinoquinoline N-methylpyrolidine 8-hydroxyquinoline
Brown:	2-hydroxypyridine, 3-hydroxypyridine, 4-hydroxypyridine 4-hydroxy-2-methylpyridine, 2-hydroxyquinoline 3-aminoquinoline, anisole, quinoline, isoquinoline
Orange:	4-hydroxy-2-hydroxymethyl-5-methoxypyridine, 2-(1-n-hexyl)pyridine
Yellow:	2-(1-n-hexyl)pyridine-1-oxide, glycine, piericidin A
No Colour:	4,6-dimethoxy-2,3-dimethylpyridine 4,6-dimethoxy-2,3-dimethyl-5-nitropyridine Acetamide, benzamide, urea, uracil, L - alanine, L - valine, L-leucine, 4-nitrobenzoic acid, adenine.

Folin-Denis Reagent[143]

Application was as a spray to dried spots of 10% solutions of test compounds.

Blue: All phenols tested including 3-hydroxypyridine and piericidin A
4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine,
4,6- " -2,5- " -3- "

No Colour: 2-and 4-hydroxypyridine, 4-hydroxy-2-methylpyridine,
4-hydroxy-2-hydroxymethyl-5-methoxypyridine

Enol Spray[216]

4% solutions of ferric chloride and potassium ferricyanide were mixed in equal proportions, and immediately applied as a spray to dried spots of 10% solutions of test compounds.

Rapidly produced blue colour reaction

All phenols tested, including 3-hydroxypyridine and piericidin A.

4,6-dimethoxy-2,3-dimethyl-5-hydroxypyridine
4,6- " -2,5- " -3- "

Some aromatic amines;

e.g. 5-amino-4,6-dimethoxy-2,3-dimethylpyridine
3- " -4,6- " -2,5- "

Brown or Green Colour Reaction turning blue only very slowly

2-, and 4-hydroxypyridine, 4-hydroxy-2-methylpyridine, 4-hydroxy-2-hydroxymethyl-5-methoxypyridine, anisole, 1-(2-pyridyl)propan-2-ol.

MASS SPECTRAL APPENDIX

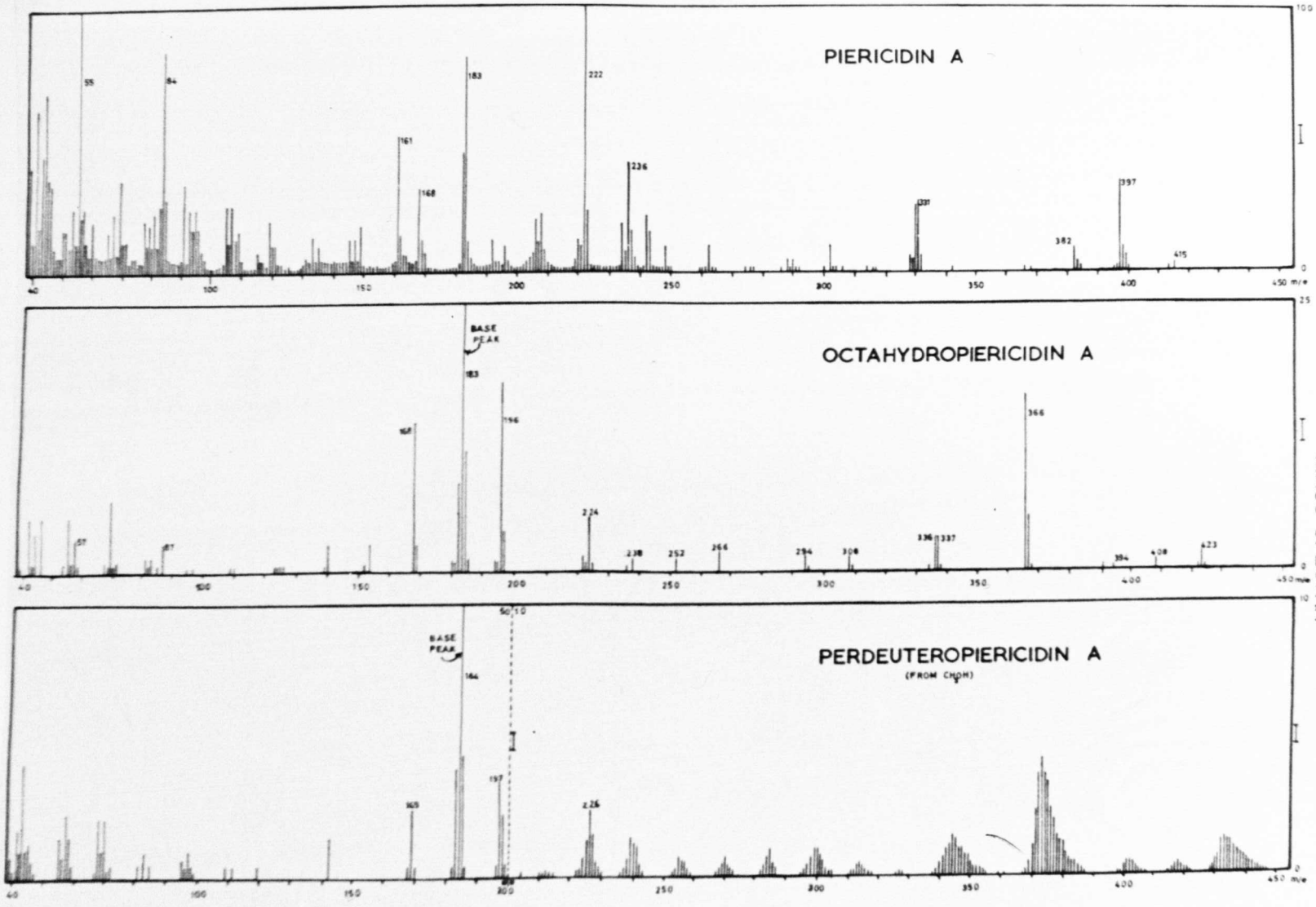


FIGURE 40

The mass spectrum of perdeuteropiericidin A from d, methanol has peaks at m/e = 170, 185, 198 and 227 in place of those at one mass unit less shown above.

FIGURE 41

MASS SPECTRA

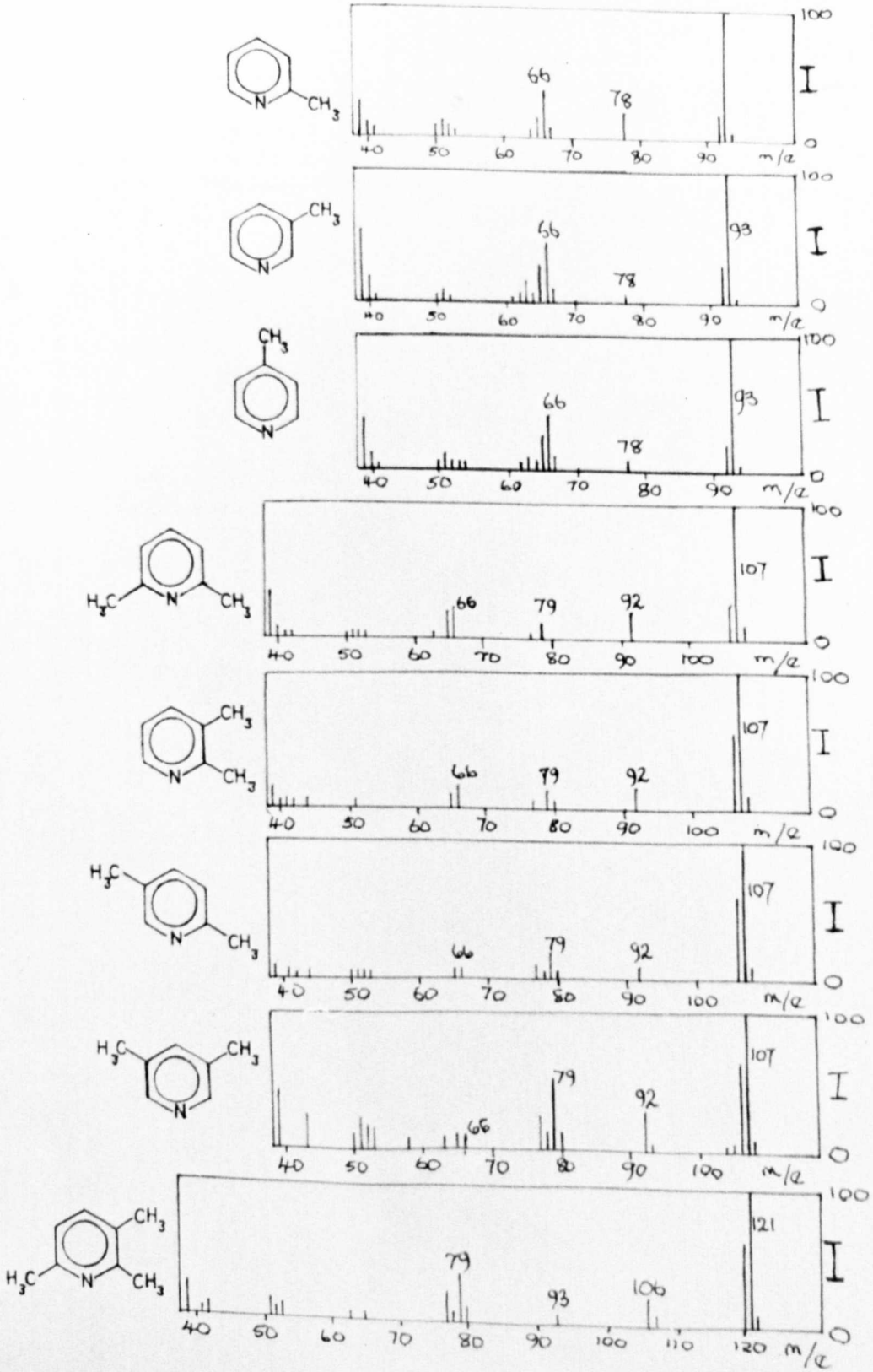


FIGURE 42

MASS SPECTRA

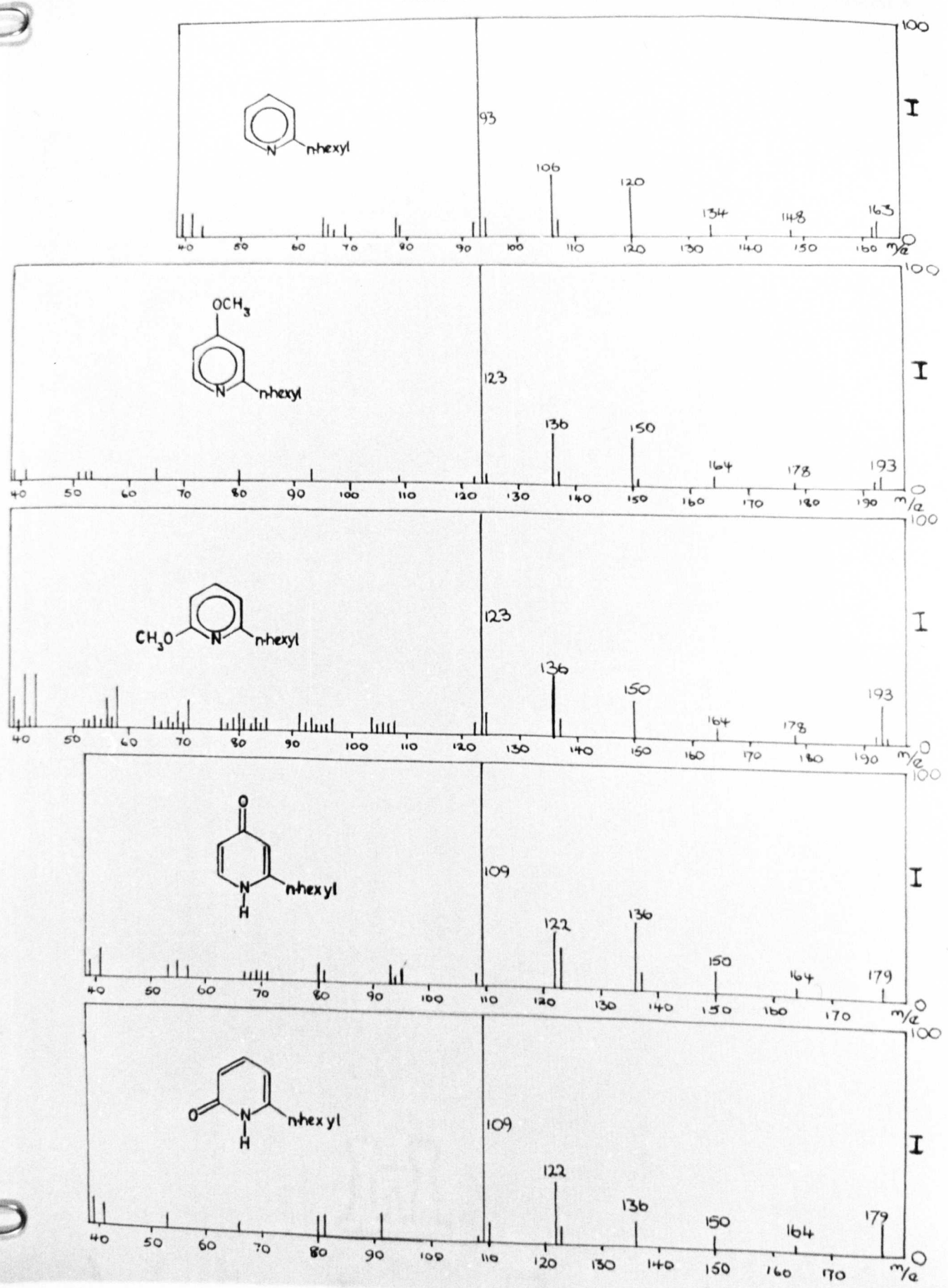


FIGURE 43
MASS SPECTRA

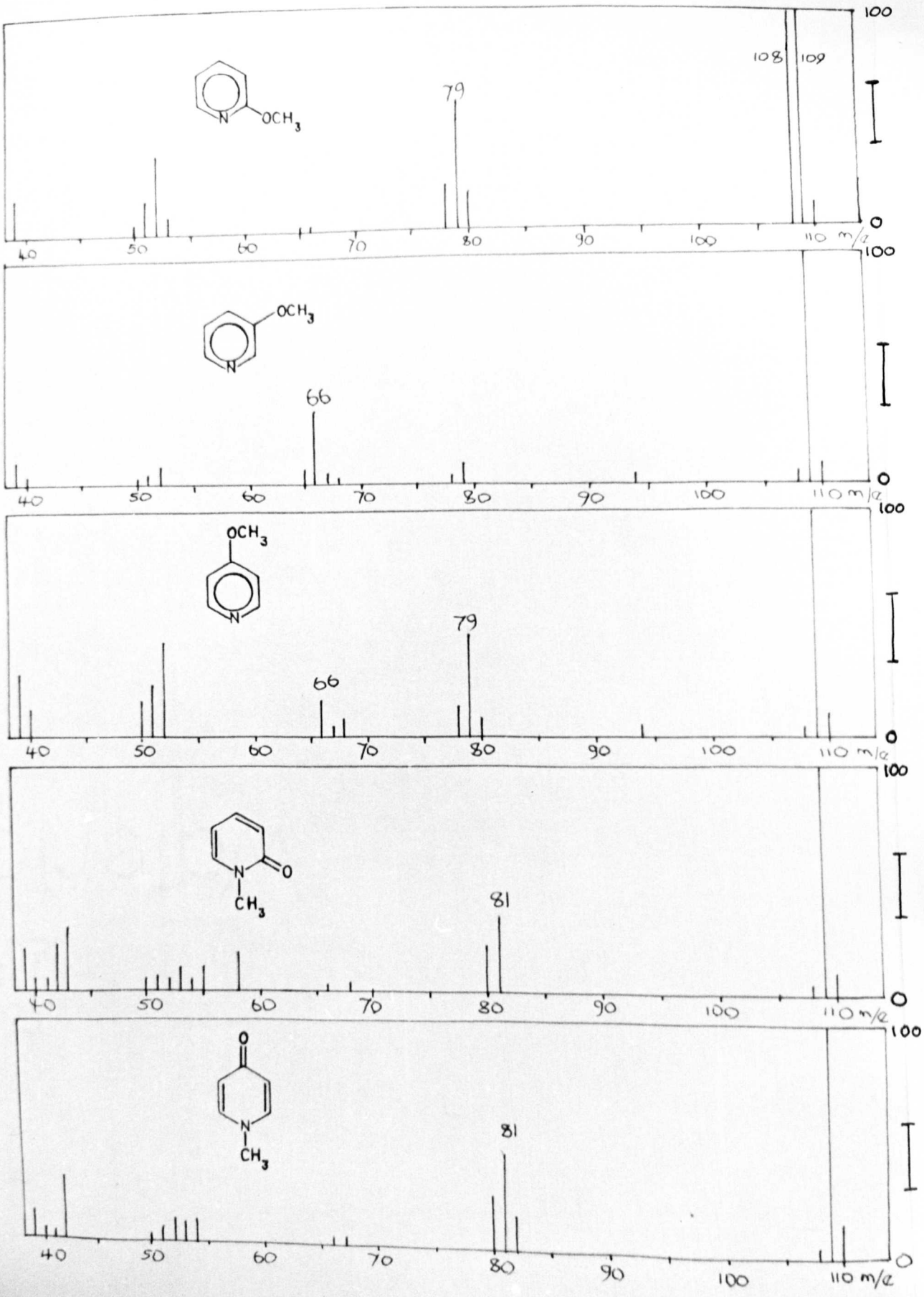


FIGURE 44

MASS SPECTRA

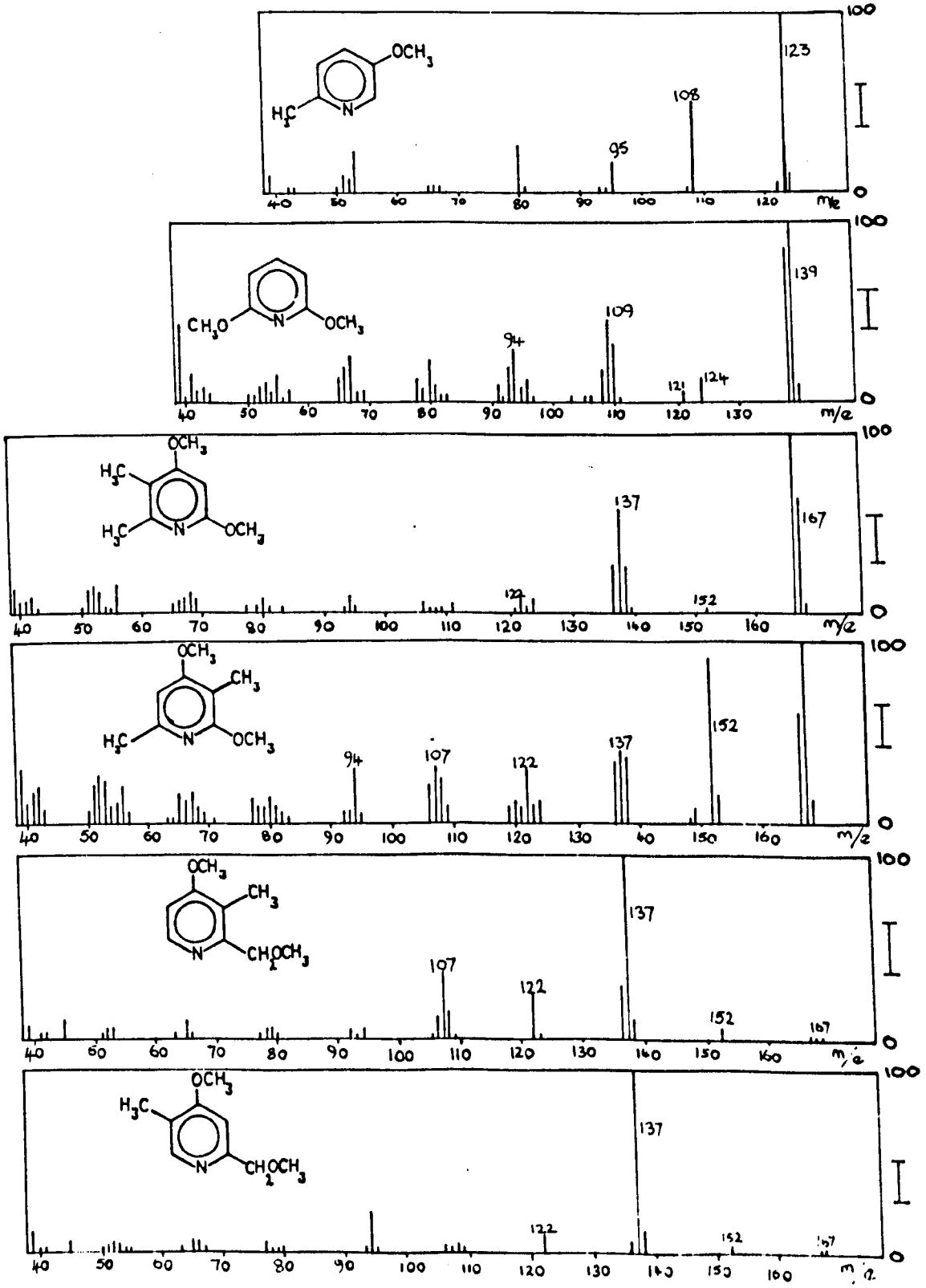
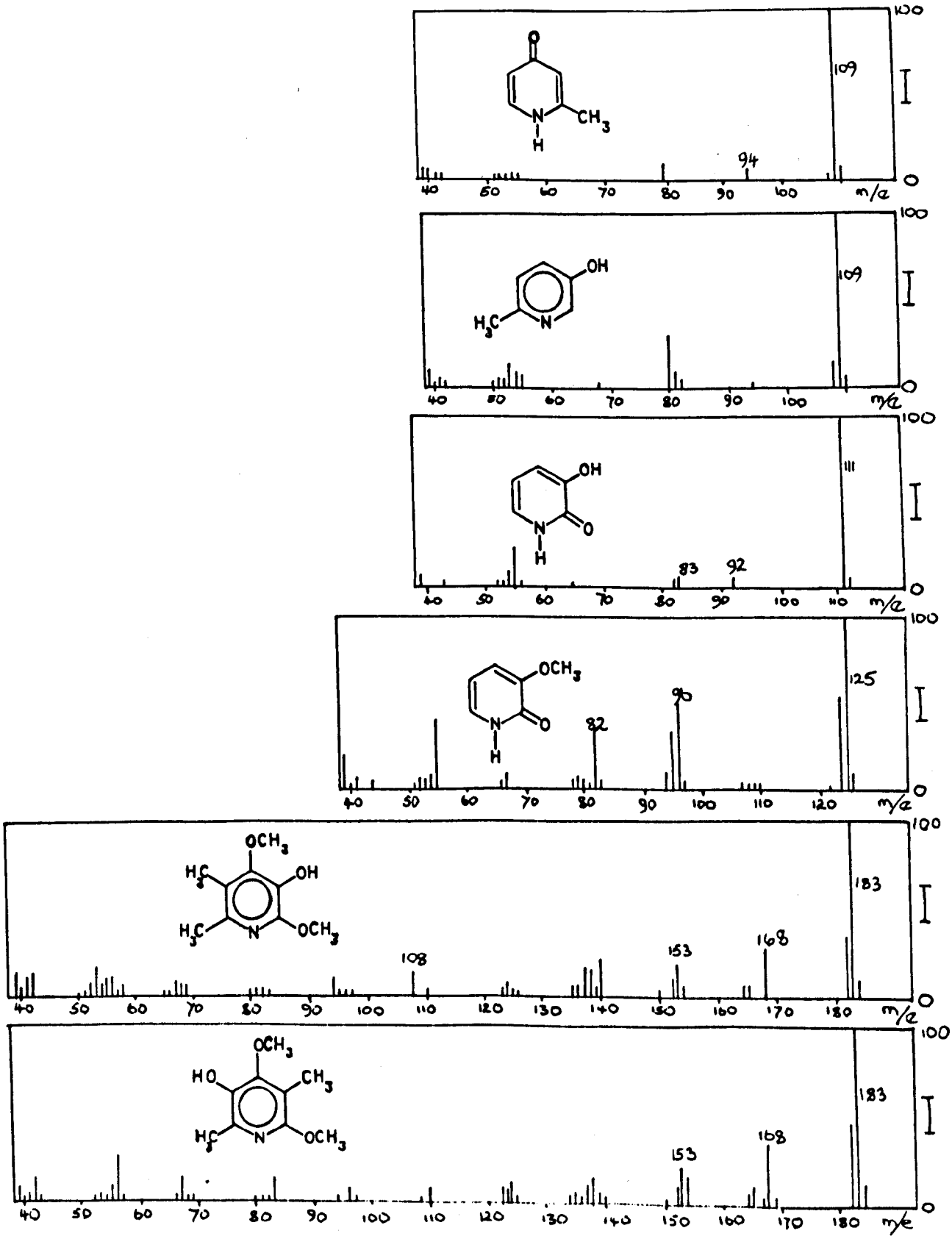


FIGURE 45

MASS SPECTRA



The structures of the ionic species represented in the following figures areproposed for the convenience of interpretation of the corresponding mass spectra. The ions are not necessarily present in such a form in the mass spectrometer.

FIGURE 46

MASS SPECTRAL INTERPRETATIONS

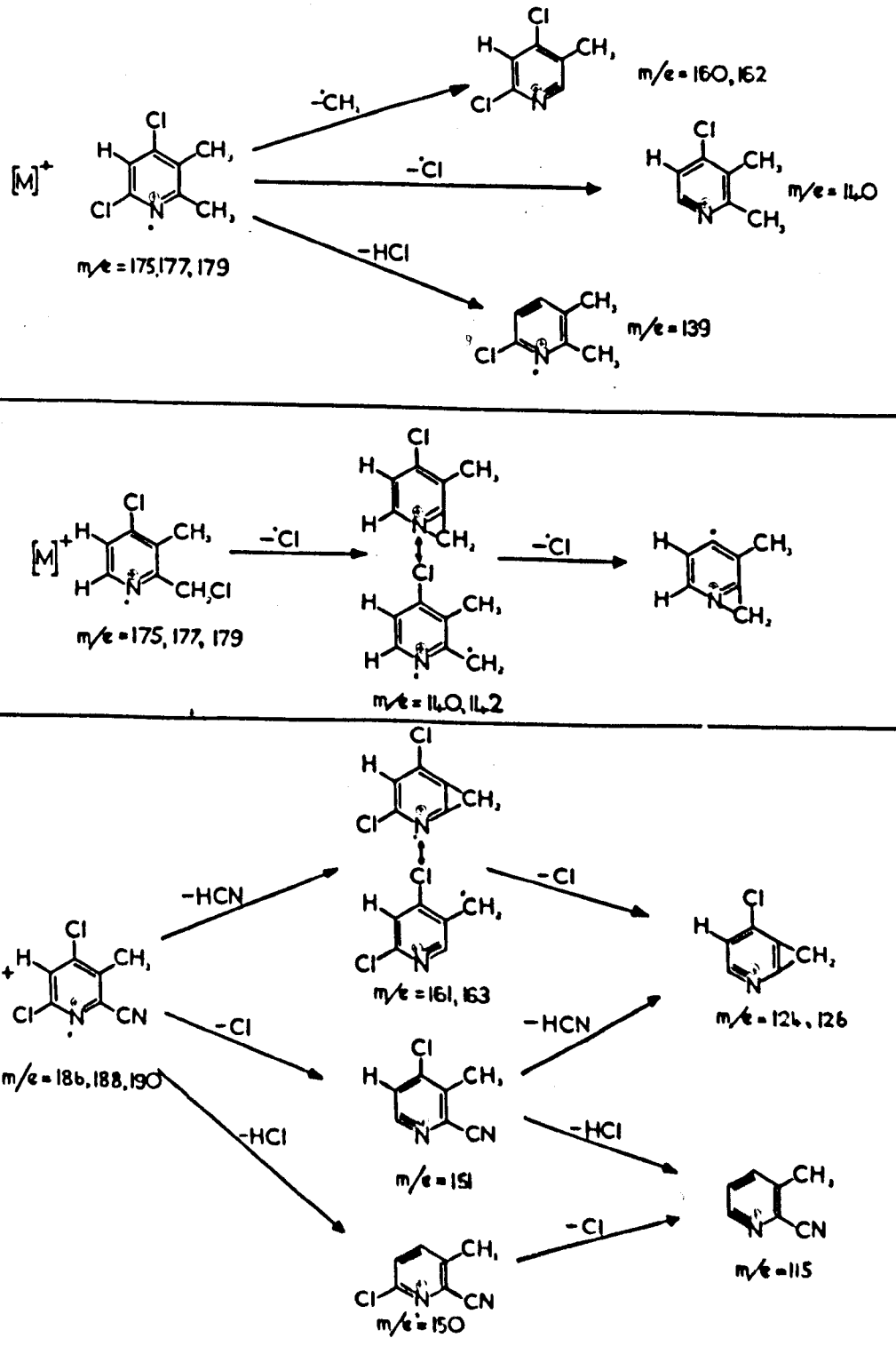


FIGURE 47

MASS SPECTRAL INTERPRETATIONS

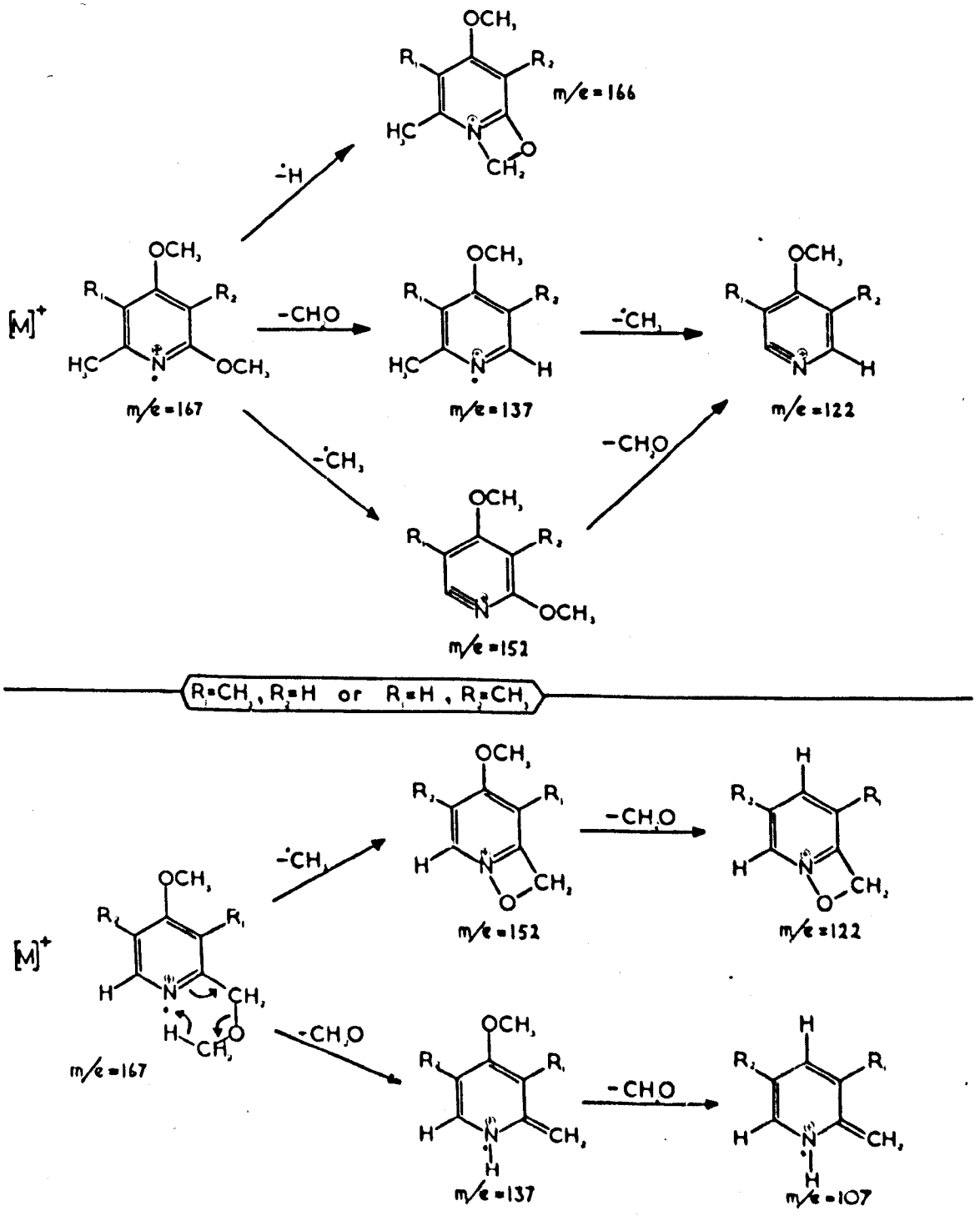
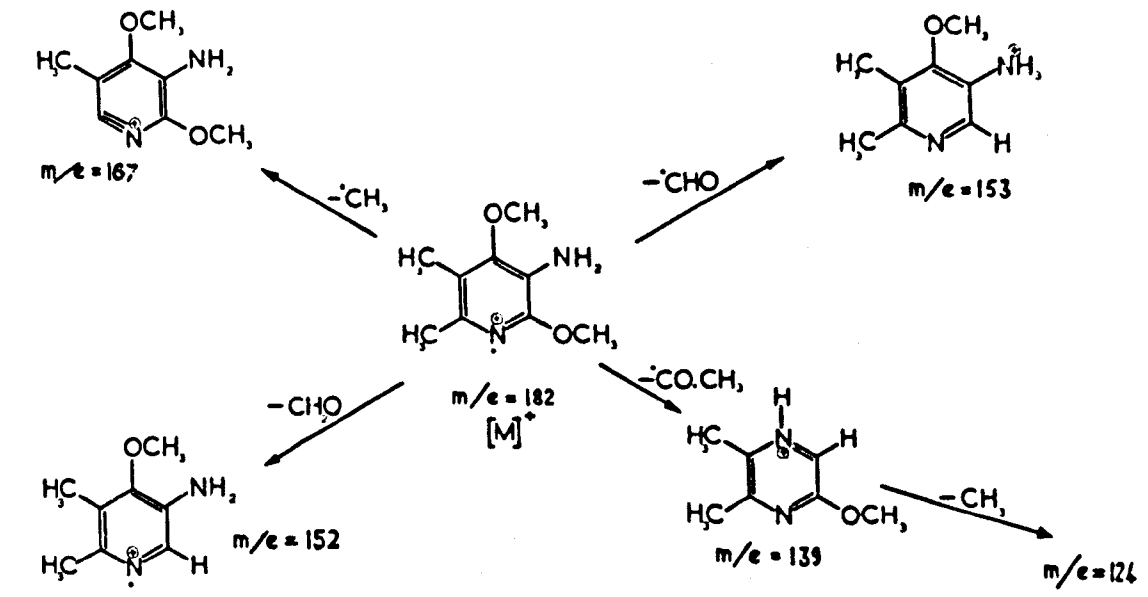


FIGURE 48

MASS SPECTRAL INTERPRETATIONS



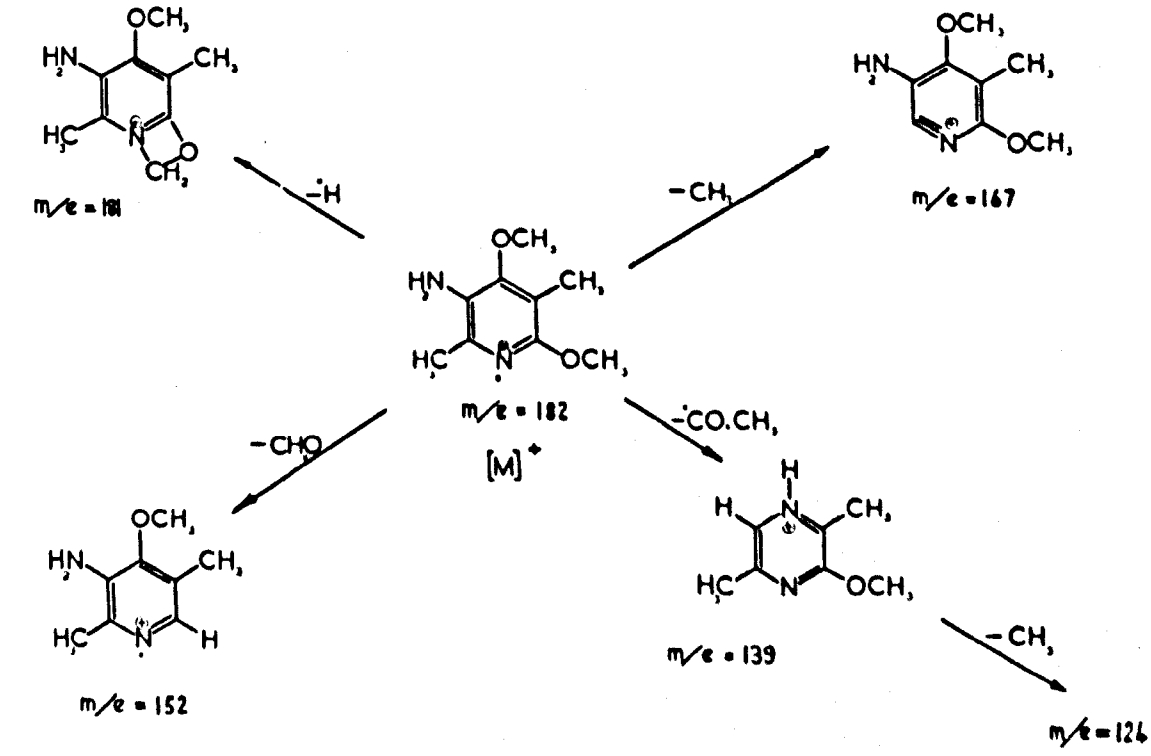


FIGURE 49

MASS SPECTRAL INTERPRETATIONS

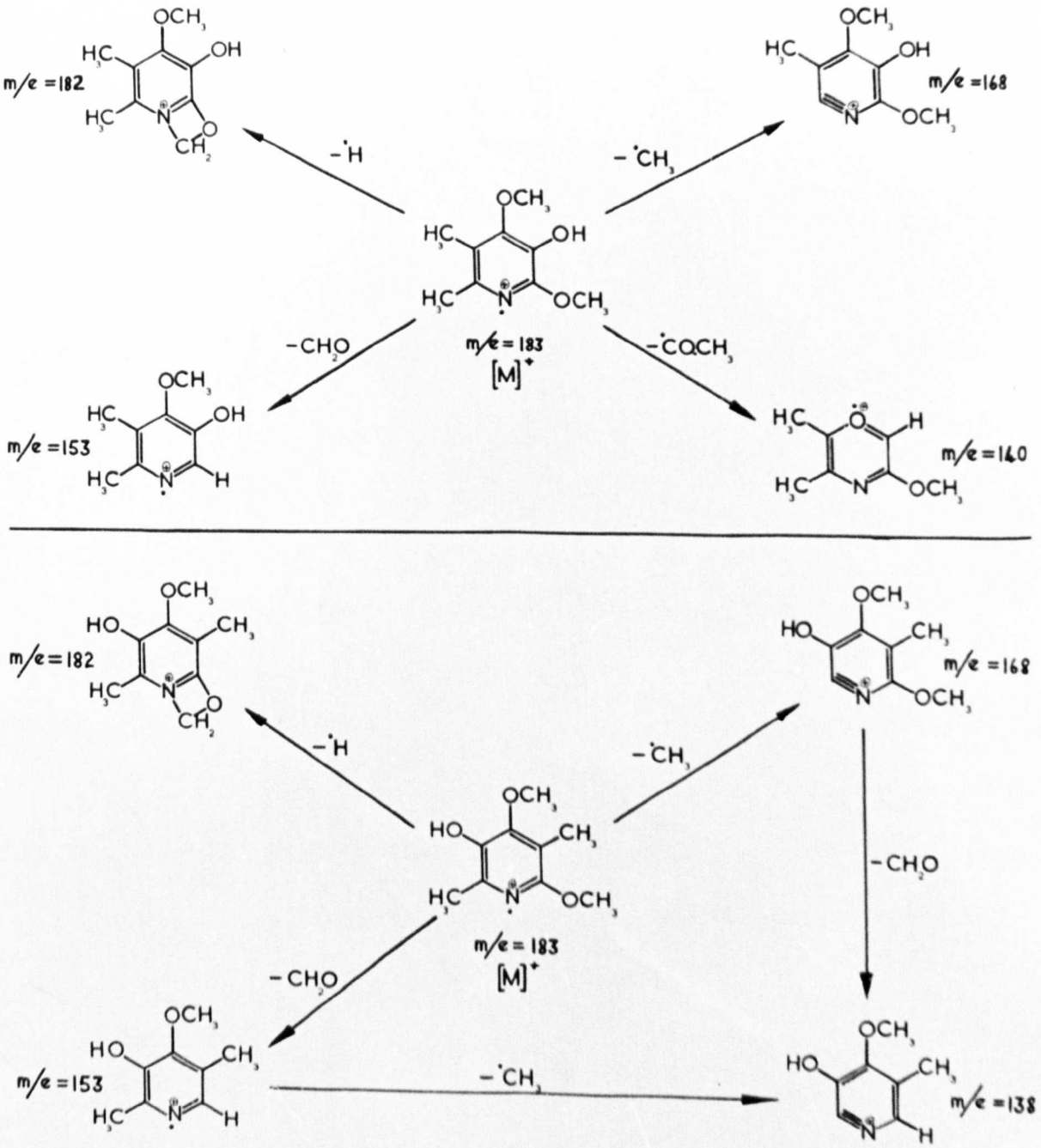
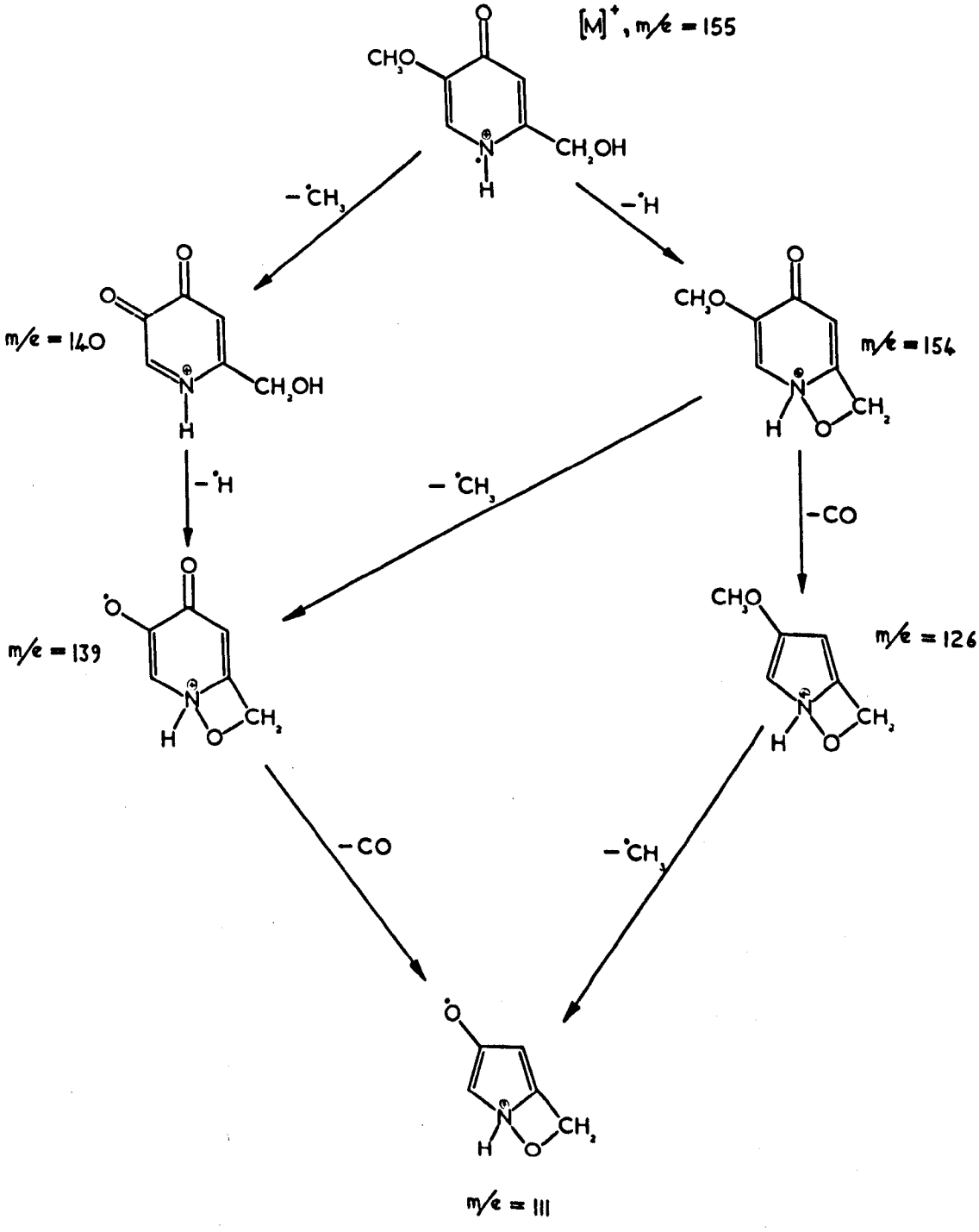


FIGURE 50

MASS SPECTRAL INTERPRETATIONS



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